INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

ProQuest Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

UMI®
Development and Characterization of a Murine Model of Autoimmune Encephalitis with the Type 2 Central Nervous Voltage-gated Sodium Channel as the Autoantigen

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

Susan E. Coulson Burghes, A.B., M.S.

The Ohio State University 2001

Dissertation Committee:
Professor Paul A. Fuerst, Chairman
Professor Kottil W. Ram Mohan, Advisor
Professor Kevin Hackshaw
Professor Thomas Prior

Approved by:

Co-Advisors
The Molecular, Cellular, & Developmental Biology Program
Multiple Sclerosis is the most common neurodegenerative disorder of the central nervous system with an incidence of 1:800 individuals of Northern European descent living in northern latitudes. The cause of the disease remains obscure, but the progression is thought to be immune-mediated. Patients exhibit varying degrees of demyelination of the myelinated nerves in the spinal cord and brain accompanied by neural degeneration and accelerated atrophy. Immunomodulatory therapies are somewhat effective, but no treatment halts the progression of the disease.

Existing animal models of experimental autoimmune encephalitis (EAE) have focused on the proteins making up the myelin sheath as the targets of immune attack. While the myelin proteins are involved in the autoimmune disease process in humans, therapies that halt myelin destruction do not alter the course of the disease. Recent spectroscopic studies indicate that significant damage to nerve axons occurs in the early stages of MS independent of demyelination.
This thesis describes an animal model of central nervous autoimmune encephalomyelitis induced by immunizing mice with a portion of a central nervous axon-specific voltage-gated sodium channel. Thirty to forty percent of mice immunized with 100µg-1mg dosages of the sodium channel fusion protein experienced severe weight loss and a variety of neurological symptoms.

Inflammation induced by immunization with the sodium channel fusion protein was visualized in the brain with histological stains and immunohistochemical techniques. All of the mice exhibiting weight loss and increasing muscle weakness displayed meningeal and perivascular inflammation of the brain. Large aggregates of mononuclear cells were also observed in the hippocampus in some animals by histological staining. Immunohistochemistry using monoclonal antibodies for immune cell subtypes revealed a majority of cells to be macrophages. T cells were present less frequently in the brain parenchyma, but were associated with blood vessels and the meninges.
To my sons, Evan and Gareth

Twenty years from now you will be more disappointed by the things you did not do than by the ones you did do. So throw off the bowlines. Sail away from the safe harbour. Catch the trade winds in your sails. Explore. Dream. Discover.

— Mark Twain
I am grateful to Dr. Rammohan for imparting his wisdom and dedication to the field of neuroimmunology and multiple sclerosis, and for his undying enthusiasm at the prospect of unraveling the mysteries of this frustrating disease.

I am very thankful that Lisa Gonzales worked so diligently as our technician, and enjoyed ‘exercising’ the mice as much as I did. I also thank Lisa for her time and ingenuity in making the data tables and some of the drawings for this thesis.

I am also deeply appreciative of the excellent technical advice and productive discussions I have had over the years with my co-workers, Dr. Thanh Le and Dr. Daniel Coovert. They were always in the right place at the right time for me.

I appreciate the training and assistance I received from the Veterinary staff of the University Animal Facilities, especially from Dr. Valerie Bergdall.

Finally I must thank my family and friends for relentlessly encouraging me to finish this work especially my Mom, Dad, my
husband, Arthur, and inlaws, Barbara and (the late) Harry Burghes without whose financial support, this would not have been undertaken. My best friend from childhood, Nancy, and the women in my Mah Jongg group have been a continual source of understanding and perspective. Finally, I remember the late Darryl R. Absolom, an avid scientist and soul of compassion, who took every opportunity to encourage me in this endeavor.
VITA

April 24, 1956 ....................................... New Haven, Connecticut, USA

1978 ......................................................... A.B. Mt. Holyoke College, MA

1979 ......................................................... M.S. Biochemistry, University of Maine, Orono

1979-1982 .............................................. Biochemist, Marine Colloids, FMC, ME

1983-1984 .............................................. Institute of Neurology, Queens’ Square, UK

1986-1988 .............................................. Hospital for Sick Children, Toronto, Canada

PUBLICATIONS & ABSTRACTS


viii

**FIELDS OF STUDY**

Major Field: Molecular, Cellular, & Developmental Biology
Specialty: Neuroimmunology
# TABLE OF CONTENTS

Abstract .......................................................................................................................ii  
Dedication ...................................................................................................................iv  
Acknowledgements .................................................................................................v  
Vita ..............................................................................................................................vii  
List of Tables ...........................................................................................................xiii  
List of Figures ..........................................................................................................xiv  
Abbreviations ..........................................................................................................xvi  

Chapters:  

1. ORGANIZATION OF THE DISSERTATION .............................................1  
   1.1 Background ............................................................................................1  
   1.2 Research Objectives ............................................................................3  
   1.3 Organization .........................................................................................4  

2. INTRODUCTION ..............................................................................................8  
   2.1 Multiple Sclerosis ..................................................................................8  
   2.2 Pathology of Multiple Sclerosis .............................................................10  
   2.3 Autoimmunity .........................................................................................18  
   2.4 Role of the Major Histocompatibility Complex  
      in Autoimmunity ..................................................................................21  
   2.5 Genetics of Multiple Sclerosis ..............................................................26  
   2.6 Genetic Studies of Murine EAE .............................................................31  
   2.7 Myelinated Nerves of the Central Nervous System .............................35  
   2.8 Structure and Physiology of Voltage-gated  
      Sodium Channels ..................................................................................38
2.9 Role of the Sodium Channels in Inter and Intracellular Communication in the Central Nervous System ................................................43
2.10 Animal Models of Experimental Autoimmune Encephalitis ..........................................................50
2.11 Epitope Spreading ..................................................................................................................................59

3. MATERIALS AND METHODS ..........................................................................................................................66

3.1 RNA Extraction ..................................................................................................................................................66
3.2 First strand cDNA synthesis ..............................................................................................................................69
3.3 Polymerase chain reaction ...................................................................................................................................70
3.4 Cloning and colony screening ............................................................................................................................74
3.5 Sequencing ........................................................................................................................................................77
3.6 Subcloning FP1 into pGEX-2TK ..........................................................................................................................80
3.7 Expression of the FP1-GST fusion protein in pGEX-2TK ..................................................................................83
3.8 Western blotting ..................................................................................................................................................85
3.9 Purification of the FP1-GST fusion protein for immunizations ..............................................................................89
   3.9.1 Lysis of the amplified FP1-GST/XL-1 bacterial cultures ................................................................................90
   3.9.2 Extraction of FP1-GST from the cell lysate .................................................................................................92
3.10 Sample preparation for SDS-PAGE .....................................................................................................................93
3.11 Preparation of FP1-GST in adjuvant for immunizations ....................................................................................94
3.12 Immunization regimen .......................................................................................................................................96
3.13 Behavioral testing ...............................................................................................................................................99
3.14 Histopathology ................................................................................................................................................101
3.15 Immunohistochemistry ....................................................................................................................................102
3.16 Immunofluorescence of the CNaIIa cell line .................................................................................................105

4. RESULTS ............................................................................................................................................................108

4.1 RT-PCR of the FP1 transcript from the CNaIIa cell line .......................................................................................108
4.2 Sequencing the FP1 cDNA TA clone ...................................................................................................................109
4.3 Subcloning FP1 into the pGEX-2TK expression vector .........................................................................................110
4.4 Preliminary immunizations of FP1-GST in BALB/c and SJL/J mice .................................................................112
4.5 Western blot analysis of the FP1-GST fusion protein and the CNaIIa cell line ...................................................116
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Genetic loci associated with EAE</td>
<td>64</td>
</tr>
<tr>
<td>4.1</td>
<td>Preliminary dosages of FP1 in complete Freund’s adjuvant (BALB/c strain)</td>
<td>149</td>
</tr>
<tr>
<td>4.2</td>
<td>Preliminary dosages of FP1 in complete Freund’s adjuvant (SJL/J) strain</td>
<td>150</td>
</tr>
<tr>
<td>A.1</td>
<td>Antibodies for western blots</td>
<td>209</td>
</tr>
<tr>
<td>A.2</td>
<td>Antibodies for immunohistochemistry</td>
<td>210</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Schematic drawing of the central nervous type 2 voltage-gated sodium channel</td>
<td>39</td>
</tr>
<tr>
<td>4.1</td>
<td>Reamplified RT-PCR product of FP1</td>
<td>144</td>
</tr>
<tr>
<td>4.2</td>
<td>DNA alignment of the FP1 RT-PCR product and the rat SCN2a template</td>
<td>145</td>
</tr>
<tr>
<td>4.3</td>
<td>Amino acid alignment of the FP1 region of the central nervous sodium channels</td>
<td>147</td>
</tr>
<tr>
<td>4.4</td>
<td>Western blot of the FP1-GST fusion protein probed with sera from immunized and control BALB/c and SJL/J mice</td>
<td>151</td>
</tr>
<tr>
<td>4.5</td>
<td>Western blots of the FP1-GST fusion protein and the full length rat type 2 voltage-gated sodium channel</td>
<td>152</td>
</tr>
<tr>
<td>4.6</td>
<td>Immunofluorescence of the CNaIlα cell line</td>
<td>154</td>
</tr>
<tr>
<td>4.7</td>
<td>SDS-PAGE of the FP1-GST fusion protein for immunizations</td>
<td>156</td>
</tr>
<tr>
<td>4.8</td>
<td>Graph of weight changes in immunized and unimmunized SJL/J mice</td>
<td>157</td>
</tr>
<tr>
<td>4.9</td>
<td>Gait test of control and FP1-immunized SJL/J mice</td>
<td>159</td>
</tr>
</tbody>
</table>
4.10 Walkway for gait testing ......................................................... 161
4.11 Histograms of individual behavioral tests .............................. 162
4.12 Graph of clinical scores attained by vector-immunized and FP1-immunized SJL/J mice ................................. 164
4.13 H & E staining of perfused brain tissue ................................. 166
4.14 Immunohistochemistry of brain tissue from FP1-immunized SJL/J mice ...................................................... 168
4.15 Immunohistochemistry and H & E staining of brain tissue from unimmunized and vector-immunized control SJL/J mice ...................................................... 170
4.16 Scatter plots of clinical and histological scores of individual mice at weekly intervals ........................................... 172
4.17 Summary graph of histological and clinical scores of selected FP1-immunized SJL/J mice ............................ 174
ABBREVIATIONS

APC       antigen presenting cell
bp        base pair
BSA       bovine serum albumin
CFA       complete Freund’s adjuvant
CNS       central nervous system
DEPC      diethyl pyrocarbonate
DTT       dithiothreitol
EAE       experimental autoimmune encephalitis
EDTA      ethylenediamine tetraacetic acid
H & E     hemotoxilin & eosin
H37RA     strain Mycobacterium tuberculosis
IgG       immunoglobulin gamma
kb        kilobase
kDa       kilodalton
MBP       myelin basic protein
MS        Multiple Sclerosis
MSH       β-mercaptoethanol
Na+       sodium
PAGE      polyacrylamide gel electrophoresis
PBS       phosphate-buffered saline
PCR       polymerase chain reaction
SDS       sodium dodecyl sulfate
TBE       tris borate EDTA buffer
TBS       tris-buffered saline
TCR       T cell receptor
TTBS      tris-buffered saline with Tween-20
CHAPTER 1

ORGANIZATION OF THE DISSERTATION

1.1 Background

Multiple sclerosis is the most common organ-specific autoimmune neurodegenerative disease (McFarlin and McFarland, 1982a & b; French-Constant, 1994; Navrikas and Link, 1996. MS is limited to the central nervous system, and despite the frequency of the disease in individuals of North European descent, the etiology of the disease is unknown (Martin et al., 1992). The pathophysiology of MS indicates that inflammation is present on a chronic or recurring basis in the brain and spinal cord (Martin et al., 1992; McFarlin and McFarland, 1982a & b). Individuals with MS often respond to immunosuppressive and immunomodulatory therapies, but there is no way to halt the disease progression (Birnbaum and Antel, 1998; Stinissen et al., 1997 review).

The target(s) of the autoimmune response in central nervous tissues are considered to be primarily the myelin proteins that make up
the protective sheath surrounding the axons of the white matter (Rivers and Schwentker, 1935; McFarlin and McFarland, 1982a & b). The result of prolonged inflammation in these tissues along with specific autoimmune attack is poor nerve conduction perhaps due to several mechanisms of nerve degeneration. Animal models of the disease using myelin proteins as the autoantigen develop a range of responses, some of which mimic MS (Levine, 1973; Levine, 1974; Raine et al., 1980; Montgomery and Rauch, 1982). However, none of the models comprises all of the characteristics of the human disease nor are therapies designed to halt demyelination effective over the long term (Mitchell, 1993; Ebers, 1994; Stinissen et al., 1997 review). Therefore, the effects of other target autoantigens in central nervous tissue must be studied.

Recent developments in the study of diversification of autoreactivities in chronic autoimmune diseases make this research timely (Topfer et al., 1995; Tian et al., 1996; Tuohy et al., 1997; Miller et al., 1997; Chan et al., 1998). In studies of other autoimmune diseases such as the myasthenic syndromes, the initial target autoantigen is the acetylcholine receptor (myasthenia gravis) or a calcium channel (Lambert-Eaton Syndrome). Patients can develop a spectrum of autoreactivities to other tissue-specific proteins during the course of the disease (Lindstrom, 1985; Ohta et al., 1990; www.neuro.wustl.edu/neuromuscular/pathol/index.htm). Indeed, this
phenomenon has also been observed in the early stages of MS and myelin-induced experimental autoimmune encephalitis (EAE), the animal model of MS (Tuohy et al., 1997, 1998). This phenomenon is called 'epitope spreading' and is believed to occur as a result of unmasking or release of other proteins in the damaged tissue (Miller, 1995).

1.2 Research Objectives and Hypothesis

Two strains of mice were selected for immunization with a portion of the axon-specific voltage-gated sodium channel based on other studies of autoimmune disease using these strains (Wekerle et al., 1994). This large transmembrane protein resides in the short, unmyelinated regions of myelinated axons in adult brain. These regions of the axon are called the Nodes of Ranvier. The type 2 voltage-gated sodium channel (SCN2a) is responsible for the propagation of the action potential along the myelinated nerves in a process called saltatory conduction (Hille, 1992).

A sodium channel has never been used as an autoantigen in the study of autoimmune encephalitis. Due to its key role in signal transduction in myelinated axons the effects of autoreactivity to this protein should be examined. Considering the fact that the disability of
patients with MS most closely correlates with axonal loss or degeneration (Trapp et al., 1998) this study provides an opportunity to study axonal encephalitis.

The \textit{in vivo} effects of immunization with an extracellular region of SCN2a in BALB/c and SJL/J mice were assessed with a battery of behavioral tests. The histopathology of the induced disease was evaluated by histology with Hemotoxilin and Eosin staining of cells in brain and spinal cord sections. The cells contributing to inflammation, when present, were identified by immunohistochemistry.

Ultimately, it is hoped that this work will shed some light on the underlying causes of ongoing disability in MS patients.

1.3 Organization

Chapter 2 is the Introduction where background from the literature on MS, features of the immune system and current concepts of how autoimmune disease occurs are summarized. Proposed mechanisms of autoimmunity are discussed in order to understand how a properly functioning immune system can give rise to autoimmune disease. MS is a complex disorder where both environmental and genetic factors are believed to play a role. A summary of genome-wide scans using microsatellite markers in humans and inbred mice is presented.
The goal of the genetic studies is to find associated genetic loci to MS and the animal model, experimental autoimmune encephalitis, EAE.

An overview of the structure of the voltage-gated sodium channel is presented, and its role in signal propagation along the myelinated axon is discussed. A summary of the current animal models of EAE is included in Chapter 2. A section is also dedicated to discussion of a phenomenon called epitope spreading which appears to be a mechanism by which MS patients and animals with EAE broaden the spectrum of autoreactivity from an initial autoantigen to other tissue-specific proteins. This observation suggests that autoreactivity to axon-specific sodium channels may exist in regions of active inflammation or autoimmune attack in the CNS of MS patients. Therefore, aspects of sodium channel-induced EAE described in this thesis complement features of existing EAE animal models.

Chapter 3 describes the methods used to clone the extracellular region of SCN2a and express it as a fusion protein. While many of these experiments can be done partially or entirely in kit form, most of the procedures undertaken here were done by traditional methods.

The resulting fusion protein served as the autoantigen used to immunize BALB/c and SJL/J mice. The effects of autoreactivity to this fusion protein in mice were assessed by a battery of behavioral tests and
ranked on a scale of clinical disability. Ongoing inflammation in central nervous tissues was examined with histological staining and immunohistochemistry. The histopathological effects of immunization with the FP1 fusion protein were also ranked on a histopathology scale described in Materials and Methods.

The fourth chapter presents the results of this investigation beginning with the cloning and expression of the FP1-GST fusion peptide. Preliminary experiments immunizing BALB/c and SJL/J mice with preliminary doses of the fusion protein in complete Freund’s adjuvant were evaluated to determine whether autoreactivity to FP1 could be induced in these murine strains. The optimal dose groups were scaled up to study the effects of FP1-GST in a susceptible strain. A substantial portion of the Chapter 4 is devoted to behavioral and histochemical analysis of the mice immunized with selected dosages of FP1 along and the control groups. The raw data from behavioral analysis was used to assign a composite clinical score to each animal at the time of the evaluation. Similarly, the types of histopathology observed in FP1-immunized animals was scored and used to assign a summary histopathological ranking for each animal. Animals were sacrificed at weekly intervals for the purpose of histological assessment. The
relationship between inflammation seen in the cerebellar and meningeal regions of the brain and behavioral analysis of FP1-immunized mice is demonstrated.

Chapter 5 summarizes the findings from this research putting them in the context of the current state of the field of central nervous autoimmune encephalitis. The future directions for this work are discussed as part of the broader theme of developing animal models of immune-mediated neurodegenerative diseases.
CHAPTER 2

INTRODUCTION

2.1 Multiple Sclerosis

Multiple Sclerosis (MS) is a disease of abnormal nerve conduction in the central nervous system (CNS) characterized by the presence of demyelinating inflammatory lesions in the brain and spinal cord (French-Constant, 1994; Prineas and McDonald, 1997). It is the most common neurodegenerative disorder of the central nervous system with an incidence if 1:800 individuals of North European descent (Davenport, 1922; Sadovnick and Ebers, 1993; Poser, 1994). The disease has variable progression ranging from a relapsing-remitting course where episodes of paralysis, sensory abnormalities and visual symptoms are frequent, and recur over a period of decades (McFarlin and McFarland, 1982a & b). There are two distinct progressive forms, primary progressive where the patient deteriorates from the outset of symptoms and a secondary progressive form. Patients with the secondary
progressive form of MS respond to therapeutic intervention, whereas the primary progressive patients do not (Birnbaum and Antel, 1998; Mitchell, 1993; Ebers, 1994).

Several theories exist as to the etiology of MS, but a specific environmental agent has never been found (Johnson, 1985; Allen and Brankin, 1993; Kurtzke, 1993). The progression of the disease is studied separately since the initiating events are unknown. However, it is generally agreed that progression is immune-mediated (Birnbaum and Antel, 1998; Wucherpfennig et al., 1991; Zhang, 1995; Martin et al., 1992).

Areas of demyelination are a classic feature of MS brain and spinal cord and these areas are typically associated with inflammatory lesions (McFarlin and McFarland, 1982a & b). The normal appearing white matter (NAWM) has been traditionally thought to retain normal function, but recent spectroscopic studies have shown that the NAWM is not normal, and may well be areas of future demyelination or axonal injury (Trapp et al., 1998; Matthews et al., 1996; Werring et al., 2000). Results from spectroscopy have also demonstrated that abnormalities seen by MRI imaging only correlate partially with the degree of patient’s disability injury (McLean et al., 1993; Davie et al., 1994; Revescz et al., 1994; Loseff et al., 1996). Not all patients who have disease symptoms have lesions visible by magnetic resonance imaging (MRI), and conversely,
asymptomatic individuals can have lesions. It is generally recognized that demyelination is not the only pathological process occurring in MS and that neither demyelination nor inflammation alone give rise to the dysfunction associated with this disease (Birnbaum and Antel, 1998; Trapp et al., 1998).

One group of investigators has found a biologically active peptide in the cerebrospinal fluid of Guillain-Barre and MS patients (Burkemeier et al., 1993). The purified pentapeptide has the amino acid sequence QYNAD (gln-tyr-asn-ala-asp), and has been found to reversibly bind to the external surface of sodium channels acting as a local anesthetic (Brinkmeier et al., 2000). The effect of QYNAD binding to voltage-gated sodium channels would be to produce a conduction block that could contribute to paralysis in these patients (Brinkmeier et al., 2000).

2.2 Pathology of Multiple Sclerosis

One of the earliest observations made from MS brain pathology was damage to nerve axons in the areas of the disease lesions (Charcot, 1868). In spite of these early observations, the patients' disability was attributed to the easily recognizable feature of demyelination (Charcot, 1868). Atrophy and Wallerian degeneration are two additional degenerative processes known to occur in MS patients and are
widespread in chronic patients (Quarles et al., 1989). Atrophy contributes to general ‘shrinkage’ of the brain (loss of brain mass), and Wallerian degeneration is axonal loss due to inflammation, or trauma and is accompanied by demyelination (Davie et al., 1995; Loseff et al., 1996). It is the actual axonal damage and eventual neuronal loss that is believed to cause the progressive disability in the patient (Ferguson et al., 1997; Evangelou et al., 2000a & b).

Techniques for visualizing and evaluating axonal loss qualitatively and quantitatively have improved over the past few years. Stains using cell type-specific monoclonal antibodies, and high resolution confocal microscopy spectroscopy has replaced analysis of tissue by silver stains (Trapp et al., 1997). The availability of reliable, axon-specific antibodies such as anti-phosphorylated and non-phosphorylated neurofilament allows high resolution microscopy to evaluate the status of axons in MS lesions (Trapp et al., 1998). Healthy axons contain primarily phosphorylated neurofilaments, which are essential in axonal transport of metabolites to the pre-synaptic region of the axon where these signals cause the release of neurotransmitters that can suppress or stimulate downstream neurons (Sanchez et al., 1996; Arbuthnott et al., 1980). Dying or injured axons have greatly increased amounts of the non-phosphorylated form of neurofilament protein, take on a tapered morphology as their caliber decreases, and conduction decreases
concomitantly indicating that normal transport processes are disrupted (Trapp et al., 1998; DeWaegh et al., 1992; Hsieh, S-T. et al., 1993; Raine, 1997).

Trapp and co-workers have determined the extent of axonal damage and neuronal loss in MS plaques from postmortem brain tissue from MS patients (Trapp et al., 1998). They developed a quantitative correlation between brain atrophy in these relapsing-remitting patients (Trapp et al., 1998) and their clinical evaluation scores (Kurtzke, 1983). These investigators found that brain atrophy is really a loss of tissue density, and since the dense material in brain is the non-myelin material, neuronal loss must be the main contributor to atrophy. Physical studies of brain volume indicate that normal brain atrophies with age, but in MS patients this is significantly more pronounced and due at least in part to neuronal loss and accelerated atrophy (Loseff et al., 1996a & b; Davie et al., 1995).

A semi-quantitative technique, MR-spectroscopy has been used to measure the relative levels of several brain metabolites that act as indicators of pathology. N-acetyl aspartate (NAA) is a well-recognized marker for neurons, dendrites and axons (Loseff et al., 1996a; Arnold et al., 1994; Davie et al., 1994; DeStefano et al., 1995; Matthews et al., 1996). In the white matter of brain, the relative amounts of NAA have been correlated with the relative density of axons. MS brain has
decreased levels NAA compared with the levels of choline and creatine (Loseff, et al., 1996b; Arnold et al., 1994; Davie et al., 1994; DeStefano et al., 1995; Matthews et al., 1996). Interestingly, the abnormal levels of these brain metabolites and signal molecules are not restricted to areas of inflammation or demyelination, but extend into the normal appearing white matter (NAWM) of patients (Matthews et al., 1996; Trapp et al., 1998; Evangelou et al., 2000a; Werring et al., 2000).

Accumulation of glutamate, the most common neurotransmitter in the brain, has been observed in regions of NAWM and results in glutamate toxicity to the surrounding tissue (Hardin-Pouzet et al., 1997; Pitt et al., 2000). These findings suggest a global disruption of cellular metabolism and signaling in central nervous tissue of MS patients (Pitt et al., 2000). Both neurons and glia fail to breakdown glutamate leading to its accumulation (Hardin-Pouzet et al., 1997; Pitt et al., 2000). Beta-amyloid precursor protein (APP) which is typically transported by fast axonal transport, accumulates in axonal processes. Some of these processes take on a tapered morphology with a bulbous end indicating that axonal transport mechanisms are not working, and that axonal transection has occurred (Ferguson et al., 1997).

Despite the early observations of Charcot and indications that the axon is the ultimate target of the disease process in MS, most research efforts have focused on the oligodendroglial cells and the myelin proteins.
Demyelination and remyelination are observed in areas of inflammation in MS brain tissue (Raine and Wu, 1993). Circulating antibodies to myelin basic protein (MBP) and MBP autoreactive T cells have been detected in MS patients and healthy individuals (Martin et al., 1992; Johnson, 1985).

Demyelinated axons respond poorly to electrical stimuli due to loss of the high resistance, low capacitance myelin sheath. Also, sodium channels are no longer clustered at high density in the nodal region, but instead are distributed along the axolemma. This rearrangement of membrane ion channels drains current from the axon without generating an action potential. Demyelinated axons reorganize their constituents. This level of plasticity contributes to clinical remission (Bostock and Sears, 1978; Foster et al., 1980; England et al., 1991). Compensatory expression of sodium channels in transgenic animals has also been demonstrated (Westenbroek et al., 1992).

What role the myelin proteins play in the autoimmune process in MS remains unclear (Birnbaum and Antel, 1998). Ironically, antibodies to MBP have been shown to promote remyelination and therefore, the exact nature of these molecules in the ultimate pathogenesis of MS is unknown (Johnson, 1985). Furthermore, autoreactive T cells to a variety of self proteins have been found in healthy individuals (Wekerle, 1992; Kojima et al., 1996; Targoni and Lehman, 1998). Their
significance in normal individuals is not understood. How or when these cells become activated also remains elusive. Theories of a late onset response to a viral infection where naïve or other potentially autoreactive T cells may come in contact with myelin as a result of a compromised blood brain barrier cannot be ruled out (Kermode et al., 1990; Sharief and Thompson, 1992; Rosenberg et al., 1996). It has been observed that if sensitization to MBP or proteolipid protein (PLP) the major component of central myelin, occurs in young adults, active disseminated encephalitis results (Stinnissen et al., 1997 review). This resembles the acute MBP-EAE induced in mice. If very young children experience infections that sensitize them to myelin, complete tolerance is achieved (Alvord et al., 1965).

The plaques are comprised of phagocytic macrophages, B cells and \( \text{T}_{\text{H}1} \) type T cells. Elevated levels of MHC class II antigens and cytokines, signal molecules involved in inflammation (Navikas and Link, 1996). The presence of activated T cells in the brain during the course of systemic viral infections have reinforced the notion that the T cells gain access to the CNS following a viral infection, possibly as a result of systemic immune activation (Johnson, 1985; Kermode et al., 1990). A link between a viral infection and the onset of MS has not been proven because a single virus is not consistently associated with the disease, and the viruses detected to date have also been found in normal subjects.
(Johnson, 1985; Kurtzke, 1993; Allen and Brankin, 1993). Also, the onset of the disease is probably years after the infection, making it difficult to trace a perpetrator.

Whether a viral infection is responsible for triggering the initial conditions, which favor MS, or are involved in the relapse phase of the disease, the progression of the disease is immunologically based. This is supported by the presence of oligoclonal bands in the cerebrospinal fluid (CSF) of patients, not found in normal subjects, and the presence of inflammatory lesions in the brains of MS patients (Lowenthal et al., 1960; Mattson et al., 1980). The possibility that MS might be a primary disorder of the immune dysregulation is a consideration, but such dysregulation is yet to be demonstrated (Birnbaum and Antel, 1998).

On the other hand, there is support for the idea that MS could result from a normal immune response, perhaps against a certain genetic background, to one or more antigens in an abnormal context (Birnbaum and Antel, 1998). The disease is responsive in varying degrees to immunosuppressive agents like interferons and immunomodulatory agents such as altered peptide ligands (Rudick et al., 1997). Therapeutic trials with the cytokine interferonγ (IFNγ) worsened the disease. This cytokine upregulates expression of tumor necrosis factor (TNFα), and is known to enhance the effects of existing inflammation (Navikas and Link, 1996).
A genetic basis for this disorder has been entertained. Genetic susceptibility is supported by the fact that certain HLA and TCR genotypes have been associated with MS in certain populations and animal models (Lando et al., 1979; Ebers et al., 1996; Haines et al., 1996; Kuokkanen et al., 1996; Sawcer et al., 1996). This facet of MS is discussed in section 2.4 of the Introduction. Traditional approaches to studying MS focused on observations of demyelination, presence of circulating antibodies to MBP, and inflammatory cells cytotoxic to oligodendrocytes. This view of MS has been overshadowed by the fact that these conditions exist in healthy individuals without active demyelination. Therefore, the function of these potentially autoreactive cells and circulating antibodies remains unknown. Not surprisingly, therapies directed at immune intervention to halt the attack on myelin have failed to provide any long lasting relief from disease progression (Stinissen et al., 1997 review). Profound loss of axons in brain lesions (75%) has been documented (Trapp et al., 1998). There is increasing evidence that pathological changes in brain tissue where myelin is apparently normal indicate broader disease pathology or perhaps are areas of future inflammatory lesions (Trapp et al., 1998; Smith et al., 2000).
2.3 Autoimmunity

Autoimmunity is thought to occur when the immune system fails to distinguish 'self' antigens from 'non-self'. The nature of immune responses to pathogens is fairly well characterized, but the mechanisms of self-tolerance have eluded full understanding of how autoimmunity to specific self-antigens can occur. That is to say, establishing self-tolerance is part of the process, and maintaining tolerance under conditions of stress, infection, and aging is not well understood. Also, why some individuals develop one or more autoimmune conditions is only beginning to be understood through genetic and epidemiological studies.

The immune system is a rapid response system, which is highly polymorphic and multi-faceted. Innate immunity consisting of circulating neutrophils and macrophages can only recognize surface molecules of pathogens that have remained structurally constant. These cells are present in vertebrates from birth, and when stimulated by the recognition of a non-host antigen, they effectively mobilize, and phagocytize the invading pathogen (Janeway and Travers, 1997). However, bacteria have evolved coats that mask antigens, and viruses are hypermutable which render them exceedingly effective at successfully invading a host (Janeway and Travers, 1997). Therefore, immune
recognition must be extremely adaptable to counter the constantly changing nature of antigen stimulation. This process of immunological adaptability is part of the host’s early development and exposure to large numbers of antigens (Janeway and Travers, 1997).

Lymphocytes express cell surface receptors of a given type, but each individual lymphocyte possesses a unique version of the antigen receptor (vonBoehmer, 1993). Only lymphocytes which encounter their cognate antigen will be stimulated to replicate, thereby expanding the population of that lymphocyte clone bearing its unique antigen receptor and triggering its development into a mature effector cell. This clonal selection process is key to lymphocyte development and the resultant immune competence of the host.

Lymphocytes come in contact with the body’s own tissues and cell surface markers from the beginning of life. Indeed, self-tolerance is established in the human fetus before birth (Janeway and Travers, 1997). Introduction of donor tissues or cells postnatally produces graft-host disease indicating that a full set of mature lymphocytes exists, and these lymphocytes can detect HLA differences between the donor and the recipient (Janeway and Travers, 1997). If, however, bone marrow cells were transplanted into a fetus, naïve lymphocytes specific for the donor
tissue antigens would still be available for selection and deletion from the host’s final repertoire of mature lymphocytes (vonBoehmer, 1993; Sprent and Webb, 1995).

Self-tolerance is acquired in humans, meaning that initially the immune system can potentially respond to self-antigens (VonBoehmer, 1993). Autoreactive lymphocytes are removed from the developing T lymphocyte pool by clonal deletion in the thymus where lymphocytes bearing tightly bound self-ligands are eliminated from circulation (Kappler et al., 1987; Sprent and Webb, 1995; Egwuagu et al., 1997). This is called central tolerance. The second mechanism for developing self-tolerance is clonal inactivation induced by tissue-specific antigens presented in the absence of co-stimulatory molecules. This process is called anergy, and it renders a T cell recognizing self-peptides refractory to activation (Arnold et al., 1993; Zal et al., 1994; Sharpe, 1995). It is also known as peripheral tolerance. This is an important process since not all potentially self-reactive lymphocytes are detectable in the thymus because they may recognize tissue-specific peptides not present in the thymus (Klein et al., 2000). Therefore, it is crucial that T cell activation only be permitted to occur in the presence of: 1) sufficient levels of antigen, 2) presented by an antigen presenting cell (APC), 3) in the presence of appropriate co-stimulatory molecules. Anergy in B cells occurs in the presence of soluble self-antigens where the self-antigen
binding to the B cell surface immunoglobulin (Ig) will not stimulate signal transduction (Fulcher and Basten, 1994). Antigen binding will also fail to stimulate Ig production by the B cell even in the presence of a T cell (Janeway and Travers, 1997). These cells fail to mature and are removed from the mature lymphoid population by being trapped in secondary lymphoid tissues and excluded from the primary lymphoid tissues. In this way, the long term B cell pool of these unreactive cells is depleted (Beverley, 1991; MacKay, 1993; Sprent and Webb, 1995; Janeway and Travers, 1997).

2.4 Role of the Major Histocompatibility Complex in Autoimmunity

The Human Leukocyte Antigens (HLA), and the murine equivalent, the Major Histocompatibility Complex (MHC), are multi-allelic genes that code for the proteins that present (ligand binding) antigenic peptides on the surface of cells. There are 2 types of MHC genes – MHC I and MHC II. MHC I is expressed on the surface of all nucleated, somatic cells except neurons and mature muscle (Janeway and Travers, 1997; Dr. Arthur Burghes personal communication). The MHC I α subunit is synthesized in the rough ER where short peptides from intracellular proteins that have been degraded by low molecular weight protease can bind in the cleft between the β1 and β2 globular domains of MHC I (Klein,
1986; Janeway and Travers, 1997). Successful binding induces a conformational change that allows β2 microglobulin, an invariant protein, to associate with the MHC I-peptide complex (Germain, 1994; Janeway and Travers, 1997). This final complex is transported to the cell surface where the bound peptide is displayed. Typically, proteins from replicating intracellular virus and proteins expressed normally by host cells, are bound to MHC I and expressed at the cell surface to maintain tolerance and tumor surveillance (Song and Harding, 1996; Janeway and Travers, 1997).

MHC II associates with peptides of exogenous (foreign) origin, and are expressed in pairs only on the cell surface of specialized antigen presenting cells (APC's). MHC II consists of an α and β subunit which associate with a third ‘invariant protein’ that stabilizes the complex (Sant and Miller, 1994). Proteins originating from invading virus and bacteria can bind cell surface MHC II directly or be ingested by macrophages and displayed as MHC II-antigenic peptide complexes on the surface of these efficient APC’s (Cresswell, 1994). The Ig-antigen complex is internalized and proteolytically degraded in B cells (Cresswell, 1994). B cells constitutively express MHC II and therefore, vesicles containing MHC II can fuse with an endocytic compartment containing the degraded foreign peptides. Peptides capable of binding between the α1 and β1 domains of MHC II will result in expression of this peptide-bearing complex on the
cell surface (Sant and Miller, 1994). There are more MHC II alleles than MHC I, and consequently, more genetic variability (Klein, 1986). It is the MHC II loci that often show genetic association with autoimmune disease (Cresswell, 1994; Andersson and Mikko, 1995).

T cell receptors (TCR's) expressed on the surface of T lymphocytes are unique and recognize individual peptide-MHC complexes. For recognition to occur, the TCR must recognize its cognate peptide-MHC complex in the presence of appropriate accessory molecule expression (Janeway, 1992; Cresswell, 1994). The long held dogma of allelic exclusion of TCR types does not appear to hold in MS. Patients have been characterized with γ-δ cells, the function of whose function is unclear (Wekerle, 1998).

Cytotoxic T cells (CTL's) expressing the CD8+ co-stimulatory molecules on their cell surface interact with MHC I-peptide complexes (Janeway, 1992). However, if the bound peptide represents a self-antigen, the CTL recognizing this complex is deleted in the thymus or rendered inactive through anergy (tolerization). If the recognition between the MHC I-peptide complex and the CD8+ CTL is productive, other cytokines and adhesion molecules will be released, resulting in the activation of the CTL, clonal expansion, and maturation into a killer CTL (Janeway and Travers, 1997). A certain number of the activated CTL
progeny will enter a dormant state as memory CTL's becoming part of the long term pool of immunoreactive cells that can mount a repeat response to their cognate antigen (Beverley, 1991; MacKay, 1993).

The mature killer T cell is guided from the site of activation in the lymphoid tissue to target cells (infected tissue) by entering the bloodstream (Janeway and Travers, 1997). The expression of cell adhesion molecules by vascular endothelial cells alters during infection (Ebnet et al., 1996). These cell adhesion molecules interact with surface markers on mature effector cells, and guide them to specific tissues where they are likely to find target cells expressing their cognate MHC I-peptide complex (Janeway and Travers, 1997). The effector cell forms a tight junction with the target cell, which allows release of cytotoxins from specialized lysozomes (lytic granules). These cytokines penetrate the lipid bilayer, and trigger apoptosis in the target cell (Janeway and Travers, 1997).

Macrophages are efficient at ingesting microorganisms. Once ingested and processed, some of the microbial proteins are presented on the macrophages cell surface as MHC II-peptide complexes. Helper T lymphocytes designated T_H1 (CD4+) T cells, can develop specific recognition of MHC II-antigen complexes through their TCR's for individual microorganisms. The principal action of T_H1 effector cells is macrophage activation that induces anti-microbial mechanisms within
the macrophages (Stout and Bottomly, 1989). This enables macrophages to damage a broad spectrum of microbial cells and tumor cells. Activated macrophages make oxygen radicals, and stimulate the expression of nitrous oxide (NO) which is antimicrobial, but can also damage healthy cells (Munoz-Fernandez et al., 1992; Fruttiger et al., 1995; Sanchez, et al., 1996). Therefore, when infection is not present, macrophages need to be inactivated which is achieved by TH1 cell expression of CD40 ligand that in turn induces IFNγ expression. IFNγ can stimulate macrophages to express TNFα and the antibody to TNFα, thus inactivating macrophages (Stout and Bottomly, 1989; Munoz-Fernandez et al., 1992).

Th2 cells, also CD4+ helper cells, but expressing other co-stimulatory molecules to distinguish them from Th1 cells, interact with the Ig-antigen or MHC II-peptide complexes on the surface of B cells (Liu et al., 1992). This allows Th2 cells co-ordinate B cell secretion of antibody directed against peptide antigens of exogenous origin, and also to conduct surveillance of tumors (Janeway and Travers, 1997). In this way, both the cellular (T cell) and humoral (B cell) portions of the immune system are linked.
2.5 Genetics of Multiple Sclerosis

Epidemiological studies of MS have demonstrated an incidence of 1:800 (0.1%) in people of North European descent (Kurtzke, 1983b). The disease is most prevalent above 40° North latitude where the peak age of onset is the second to fourth decade of life (Kurtzke, 1983b). Factors contributing to the development of the disease have exerted their effect by the age of 15 meaning that if a person moves to a southerly clime prior to 15, it is possible to avoid the onset of the disease. After age 15, migration has no effect on the outcome (Dean and Kurtzke, 1971; Kurtzke, 1983b). These observations are considered an indication that environmental factors have an effect on the incidence of MS.

MS is believed to have both a genetic and an environmental component. Genetic data indicate that this is a complex autoimmune trait (Ebers and Sadovnick, 1994; Sadovnick and Ebers, 1993; Ebers et al., 1995) where interactions between susceptibility genes and environmental factors may play a role (Sadovnick and Ebers, 1993). Geographic studies of disease support, but are not fully consistent with a higher incidence of the disease in northern climes and diminishing towards the equator (Sadovnick and Ebers, 1993; Bulman and Ebers, 1992). There is typically a higher rate of the disease in communities with a significant Scandinavian population (Davenport, 1992; Ebers and
Sadovnick, 1994). On the other hand, African Americans have approximately 50% the rate of occurrence compared to Caucasians (Ebers and Sadovnick, 1994). Some ethnic groups appear to be 'protected' from MS by having an essentially zero rate of incidence. These low incidence populations include: American Indians, Lapps of Northern Scandinavia, Hungarian gypsies, Hutterites, and Hispanic Americans living in California (Ebers and Sadovnick, 1994).

Genetic studies of autoimmune disease in humans are difficult to conduct because they often involve a number of genes whose effect may be interdependent. Also, the ascertainment of families with multiple affected sibs that fit standard criteria can be particularly difficult since age of onset and appearance of certain diagnostic features may not be temporally linked. Twin studies have demonstrated 30% concordance in monozygotic twins and 4% concordance in dizygotic twins, which is the same rate as for siblings (Ebers et al., 1986; Kinnunen et al., 1988; French Research Group on Multiple Sclerosis, 1992; Sadovnick et al., 1993). Concordance rates in twins for single gene disorders are considered a measure of the strength of the genetic effect (Ebers and Sadovnick, 1994). However, for autoimmune diseases that are generally polygenic, the concordance rates may be a better indicator of the number of genes contributing to the effect (Ebers et al., 1995).
Genetic studies using RFLP analysis of the T cell receptor repertoire (TCR) showed overrepresentation of TCRV\(\beta\)8, V\(\beta\)11 and C\(\beta\) associated with increased risk of MS in a North American population (Beall et al., 1989). However, these findings were not borne out in a Swedish study (Hillert et al., 1991). Similar studies were conducted for the TCR\(\alpha\) locus in Californian and Australian populations indicating that TCR V\(\alpha\)12.1 and some polymorphic C\(\alpha\) alleles are also associated with increased risk in those populations (Oksenberg, et al., 1989).

Four major studies involving whole genome scans of families with affected sibs have been done in Canada, the U.S., the U.K. and Finland (Ebers, G. and Canadian Collaborative Study Group, 1995; Haines et al., 1996; Sawcer et al., 1996; Ebers et al., 1996; Kuokkanen et al., 1996). Microsatellite markers spaced a maximum distance of 12-15cM were used in single point, multipoint, affected sib pair, unaffected sib pair analyses and family association studies (ibid). Additional fine mapping was done in the Finnish study based on mouse syntenic groups for EAE loci using markers spaced 4.5cM (Kuokkanen et al., 1997). Approximately 88-95% of the genome was excluded in these studies adhering to stringent criteria (Ebers et al., 1996; Sawcer et al., 1996; www.mgh.harvard.edu/depts/molneur/jh-lab.htm).

A recurring problem for investigators was low lod scores even for loci previously reported to be associated with MS and other autoimmune
diseases. Lod scores less than 3.0 would not be considered significant in
gene-trait model analyses, but they could not be excluded in these
studies because most other loci were negative values of Z. These low
scores are thought to be due in part to the reduced power of the
statistical methods employed in these studies (Lander et al., 1994;
Pericak-Vance, 1996; The Multiple Sclerosis Genetics Group, 1996). For
a locus to be considered associated with MS, it had to show evidence of
association by at least two statistical methods. Replication of results was
another difficulty because it was necessary to have approximately 60%
more material to replicate the existence of a locus than was used for the
initial detection of the locus (Suarez et al., 1994). This was an important
criterion as false positives would be detected and eliminated this way.

The only locus that has shown consistently positive lod scores in
all the human studies is chromosome 6p21 (D6S273). This is the HLA II
gene locus which had previously been studied by HLA typing of MBP
autoreactive T cells showing the most frequent haplotype associated with
MS is HLA DR2,DQw1,Dw2 (Ebers et al., 1996; Haines et al., 1996;
Sawcer et al., 1996; Kuokkanen et al., 1996). Other chromosome 6p loci,
D6S273 and D6S461 flank the HLA locus, but are non-HLA genes (Ebers
et al., 1996; Kuokkanen et al., 1996). The extended region has been
ordered as follows: D6S273-0.5cM-TNFα-0.5cM-HLA/DR-D6S461 with
TNFα showing the lowest association in this region (Z max= 0.86;
Kuokkanen et al., 1996; Kuokannen et al., 1997). Lod scores for the HLA II locus range between 2.6 and 4.6 depending on the population studied and the method of analysis (Ebers et al., 1996; Haines et al., 1996; Sawcer et al., 1996; Kuokkanen et al., 1996; Kuokannen et al., 1997).

The flanking non-HLA loci (D6S461) are under further investigation (Ebers et al., 1995; Ebers et al., 1996; Kuokannen et al., 1997). There is strong evidence in several populations that a linked non-HLA locus showing strong association with MS exists in this region (ibid). The associated eae loci from murine genetic studies are compared with their human counterparts in Table 2.1.

Other chromosomes that have shown positive scores in some populations are 1q22-23, 2q23, D3S1261, D5S416 (Finland), D5S406 (Canadian), 7q21-22 (U.S.), 11q13, 17q (U.K.), Xq13.2-22 (Canada, Finland, U.K.) (ibid). Associations previously reported such as MBP on chromosome 5, and TCRα and the Ig heavy chain loci on chromosome 14, and the TCRβ on chromosome 7 showed no association in some populations (Haines et al., 1996). The X chromosome association in the U.K. study was only observed in affected brother pairs, and may be a stochastic effect (Sawcer et al., 1996). However, X chromosomal association was also observed in the Finnish study (Kuokkanen et al., 1996). Although the authors do not speculate on a specific candidate gene in the interval Xq13.2-22, the myelin protein PLP is known to reside
at Xq22 (www.ncbi.nlm.nih.gov). Interestingly, the 2q23 (Z_{MAX}=0.73) locus contains the CNS voltage-gated sodium channel genes including SCN2a, the autoantigen used in the studies presented in this thesis. Fine mapping of the Finnish families have shown genetic association of MS with MBP and HLA-DR2, but this was observed in the Finnish cohort and not substantiated in other populations (Bell and Lathrop, 1996; Kuokkanen et al., 1997).

The investigators in all these studies concur that many of their reported associated loci have weak lod scores, but they have been unable to exclude these loci for lack of any strong associations at any other loci except chromosome 6. Their conclusion is that several genes, perhaps ten or more, exert either a weak or equal effect on MS (Ebers and Sadovnick, 1994). Therefore, the individual strength of the genetic effect at any one locus will be small, as indicated by the low lod scores. Even the chromosome 6 contribution to the disease is only about 10% of the overall genetic effect (Ebers and Sadovnick, 1994).

### 2.6 Genetic Studies of Murine EAE

Animal models of complex diseases are far more amenable to manipulation and genetic analysis. The murine model of MS, EAE, is a genetically directed disorder, and has been studied for different mouse
strains (Levine and Sowinski, 1973; Bernard, 1976; Lando et al., 1979; Baker et al., 1995). A whole genome scan has been done for the largest segregating population for an organ-specific experimental autoimmune disease with SJL/J x B10.S/DvTe mice (Butterfield et al., 1998). The SJL/J strain is extremely susceptible to EAE induction with any myelin autoantigen (Klein et al., 1983). The B10.S strain is extremely resistant to EAE (Komgold et al., 1986; Binder et al., 1993). Interestingly, both strains carry the H2^a (eae1) allele thought to be associated with EAE incidence (Klein et al., 1983). The dominant TCR determinant found in rodent EAE models is Vβ8.2. The SJL strain is deleted for this portion of the TCR gene repertoire, and therefore uses a more diverse set of TCR genes in response to challenge with myelin autoantigens, in particular Vβ12 (Binder et al., 1993).

The F1 and F2 intercross progeny of the parental strains were analyzed with microsatellite markers a maximum distance of 13-25cM apart (Butterfield et al., 1998; Deitrich et al., 1996). Most studies of susceptibility and severity of EAE are done by immunizing mice with purified autoantigens or encephalitogenic epitopes of these autoantigens (Jansson et al., 1991). The animals bred in this study were immunized with 1mg whole spinal cord homogenates in complete Freund’s adjuvant at Day 0 and Day 7 (Yasude et al., 1975). This approach obviated the need for Pertussis toxin injections to permeabilize the blood brain barrier.
(Korangold et al., 1986). Since no single autoantigen is known to be responsible for MS in humans, the use of whole spinal cord homogenates does not exclude any component of CNS tissue that may contribute to development of the disease (Butterfield et al., 1998). Furthermore, while F1 progeny may pick up dominant effects in this cross, the F2 intercrossed progeny should reveal recessive and interacting loci (Butterfield et al., 1998).

Individual loci were scored not only for association with EAE, but also analyzed as to their role in disease expression. For example, eae1-3 are associated with the incidence of EAE in this cross, but have little effect on the duration of the disease (Butterfield et al., 1998). In contrast, eae4-6 both effect the incidence of the disease and index of the disease exhibited by the animals (Butterfield et al., 1998). Averaging the clinical scores for each animal over the course of the experiment generated a susceptibility index (Butterfield et al., 1998). A severity index was determined by averaging clinical scores for each animal over the duration of the symptoms (Butterfield et al., 1998). The investigators also determined the mode of inheritance of each locus. They demonstrated that some alleles of a single locus exert a dominant effect, others a recessive effect. Locus eae9 exerted a heterozygous effect in conjunction with the dominant alleles of eae6 and 7 by shortening the duration of the disease (Butterfield et al., 1998). A summary of eae loci
either confirmed or reported in this whole genome scan is listed in Table 2.1 along with the human syntenic region and candidate genes at that locus.

The authors point out that their analysis of the role of eae1 (the H2 locus) indicates that the MHC II genes in the 2 parental strains are identical in their exonic and cDNA sequences. Therefore, no structural differences exist between these MHC alleles in the resistant and the susceptible parent strains (Butterfield et al., 1998). Furthermore, the authors state that they believe the genetic effect associated with the MHC II locus is really associated with eae5, a locus reported in this study (Butterfield et al., 1998). They find that eae5 maps just distal to MHC II and is linked to the MHC locus, but is a non-MHC encoding region (Butterfield et al., 1998). This is reminiscent of findings for the human syntenic loci on chromosome 6p (Section 2.5).

It is noteworthy that the marker selection for the human study of Finnish families was based on the syntenic groups identified in murine studies (Kuokkanen et al., 1996; Kuokkanen et al., 1997). Furthermore, the eae2 and 3 loci reported to be associated with MS in the Finnish families were not reproducible in other MS populations. This is believed to be due to the inbred nature of the Finnish cohort (Kuokkanen et al., 1996; Ebers and Sadovnick, 1994).
2.7 Myelinated Nerves of the Central Nervous System

The axons of nerves are the highways of the nervous system. They are single, tubular processes of varying length that extend from the nerve cell body sometimes through several layers of cells or tissues, and can be as long as a meter (Matthews, 1998; Hall, 1992). At the end of the axon is a bulb, which constitutes the presynaptic portion of the synaptic junction. Incoming stimuli from dendrites to the nerve cell body are converted to electrical stimuli, which trigger the opening of ion channels located at specific points along the axonal membrane (Hall, 1992; Hille, 1992). The myelinated axons are ensheathed by processes extending from oligodendrocytes in the central nervous system (CNS), and wrapping around segments of one or more axons. A single oligodendrocyte can ensheathe parts of several axons, and the sheath itself is discontinuous along the length of the axon (Hall, 1992). The processes of the oligodendrocytes consist of a complex of lipidic proteins called myelin. This discontinuous covering acts as an insulator such that electrical stimuli will only depolarize the naked, uninsulated portions of the axon. This makes nerve conduction more rapid and efficient. The unmyelinated points along the axons are called the nodes of Ranvier. Depolarizing electrical stimuli are transferred from node to node producing saltatory conduction along the axon (Hille, 1992).
Axonal structures involved in saltatory conduction of action potentials are clustered in the unmyelinated portion of the axon called the nodes of Ranvier (Kandel et al., 1991; Hille, 1992). The voltage-gated sodium (Na+) channels are present in the axonal membrane at the nodes of Ranvier where they open in response to depolarizing electrical stimuli thus generating the nerve action potential (Hodgkin and Huxley, 1952; Hille, 1992). This in turn depolarizes the axonal membrane at the node allowing a rapid influx of Na+ into the axon. Voltage-gated Na+ channels are typically fast inactivating meaning they close rapidly following the influx of Na+ ions (Catterall, 1988; Hille, 1992). Propagation of the impulse along the axon to the synapse involves activation intracellular phosphorylation cascades that effect axonal transport mechanisms (DeWaegh et al., 1992; Sanchez et al., 1996; Trapp et al., 1998).

Successful signal transduction results in either stimulation or inhibition of downstream neurons (Hille, 1992; Pitt et al., 2000). Sodium channel signal transduction and cell-cell communication pathways are discussed in detail in the Discussion (Chapter 5). Also, a variety of transport proteins and filaments are affected by ion channel signal transduction, and these contribute to the overall health, size (caliber), and conduction speed of the axon (DeWaegh et al., 1992; Sanchez et al., 1996; Trapp et al., 1998).
Myelination of axons is a developmentally regulated process that affects the maturation and health of the neuron and intracellular transport and signal processes (DeWaegh et al., 1992; Fruttiger et al., 1995; Sanchez et al., 1996). It is also has structural consequences in that certain proteins such as potassium and calcium channels are localized in the axolemma (axon cell membrane) beneath the myelin sheath in the paranodal region. These ion channels are responsible for repolarization of the membrane (Ritchie and Rogart, 1977; Catterall, 1986; England, 1990).

The demyelination observed in MS is often paranodal and has been shown to cause a redistribution of sodium and other ion channels along the demyelinated segment (Bostock and Sears, 1978; England et al., 1991; Hsieh et al., 1992). Nerve conduction in poor in these areas was originally thought to be solely due to a pathological process affecting myelin. However, there is strong support from animal mutants and models of demyelination that the axon itself may be damaged either by immune attack or by ensuing inflammation and that myelin loss is more a consequence of axonal disease (Waxman, 1977; Westenbroek et al., 1992; Trapp et al., 1998). Ultimately, conduction is lost in areas where inflammation and demyelination are present (MacDonald, 1974. Brain
lesions with both active and chronic inflammation show as many as 75% of the axons lost or damaged compared with healthy individuals (Trapp et al., 1998).

2.8 Structure and Physiology of Voltage-gated Sodium Channels

The central nervous type 2 voltage-gated sodium channel (SCN2a) is a heterotrimeric complex of glycosylated transmembrane proteins. The pore forming α subunit is a 260kDa transmembrane protein expressed at the nodes of Ranvier in adult brain. In humans, the gene for SCN2a is found on chromosome 2 (SCN2a, 2q22-q24; mouse chromosome 2), in tandem array with the genes for types 1 and 3 central nervous voltage-gated sodium channels (www.ncbi.nlm.nih.gov; Noda et al., 1986a). These large α subunits are well conserved and are approximately 6,800 bp in length (Noda et al., 1984). The type 1 channel is expressed at the axon hillock, the point where the nerve cell body elongates becoming the axonal process (Beckh et al., 1989; Gordon et al., 1987; Scheinman et al., 1989; Westebroek et al., 1989). The type 3 channel is a fetal form that is predominantly replaced by the type 2 channel in the adult (ibid). This large α subunit is heavily glycosylated, making up almost 30% of
molecular mass of this subunit on SDS polyacrylamide gels. The carbohydrate chains consist of N-linked sugars that are largely sialylated (Catterall, 1986; Hille, 1992).

![Schematic Drawing of the Central Nervous Type 2 Voltage-Gated Sodium Channel](image)

**Figure 2.1.** Schematic Drawing of the Central Nervous Type 2 Voltage-Gated Sodium Channel

The α subunit of the voltage-gated sodium channel. The portion in red (designated by the arrow) corresponds to the cloned FP1 region. FP1 is the extracellular region joining segments 5 and 6 of Domain I [D1(S5-6)]. The points designated P are putative sites of tyrosine phosphorylation; the forks are potential sites of N-glycosylation and Sc-Tx is the α-scorpion toxin-binding site (Catterall, 1992).

The pore forming α subunit is able to carry out all the electrophysiological functions of the heterotrimeric channel complex (Hartshorne et al., 1984; Sumikawa et al., 1984; Goldin et al., 1986). The hallmark secondary structure of Na+ channel α subunits is the four
repeating clusters (DI-IV) of 6 transmembrane segments (S1-6) (Noda et al., 1984; Catterall, 1988). The four repeat domains form a 3-dimensional pseudoasymmetric array in the cell membrane thus creating the channel pore (Stuhmer et al., 1989; Catterall, 1988). The transmembrane segments are a mixture of α helix and β sheet structures (Noda et al., 1989; Hille, 1992). The extended conformation of the β sheets protrude at the intracellular and extracellular surfaces of the cell membrane allowing access to the surrounding milieu (Hille, 1992). The extracellular mouth of the channel has exposed carboxyl groups, which can bind neurotoxins such as the α-scorpion toxin, saxitoxin, and the puffer fish toxin, tetrodotoxin (Vassilev, et al., 1988; Stuhmer et al., 1989). These toxins compete with Na+ for entry into the pore and slow channel inactivation (Hille, 1992). Complete and irreversible inactivation of the channel is caused by proteolytic digestion of the intracellular surface of the pore indicating that inactivation must involve a transmembrane conformational change (Stuhmer et al., 1989; Hille, 1992).

Another characteristic feature of Na+ channels is the rapid inactivation times of these channels. Sodium channel activation is caused by depolarization of the cell membrane, and is a transient event (Catterall, 1992; Hille, 1992). The reason that activation is transient is because prolonged activation causes a transition to an inactive state
rendering the channel refractory to activation by depolarization \textit{(ibid)}.

The inactivation gate of the channel is a cytoplasmic segment that connects Domains III and IV (Vassilev et al., 1988; Stuhmer et al., 1989). A group of 10 highly conserved positive and hydrophobic amino acids are responsible for the fast inactivation properties of Na+ channels. This conserved motif is thought to act as a "hinged lid" which undergoes a conformational change and closes over the intracellular mouth of the channel (West et al., 1992). This is the moiety that is cleaved by the proteolytic digestion rendering the channel irreversibly inactive as described in the previous paragraph.

The voltage sensor is located in S4 of each of the four domains and consists of repeat motifs of a positively charged amino acid (usually arginine) followed by two hydrophobic residues (Stuhmer et al., 1989). Mutation of one or more of these residues alters the voltage dependence or gating of the channel (Stuhmer et al., 1989).

Sodium channel \(\alpha\) subunits are potentially phosphorylated on any of five tyrosine residues present in the large intracellular region between Domains I and II by cAMP-dependent protein kinase A (PKA) (Kalman et al., 1990). The net effect of phosphorylation is an alteration of steady state voltage dependence of inactivation to more negative voltages without affecting the time course (Costa, 1987).

Phosphorylation is also involved in intracellular signal transduction.
involving G proteins and phosphatidyl inositol release and Ca$^{++}$ mobilization (Lakshmanan et al., 1978; Sigal et al., 1988). Activation of these pathways by brain Na$^+$ channels is thought to be involved in growth factor and cell adhesion molecule expression which affect long term modulation of the channels, their cellular localization, and cytoskeletal organization (Isom et al., 1995; McCormick et al., 1998).

In vivo, the sodium channel is a heterotrimer consisting of the large $\alpha$ subunit and 2 smaller auxiliary subunits, $\beta$1 and 2. The $\beta$1 subunit is 36 kDa in its glycosylated form (SCN1b, human chromosome 19) (www.ncbi.nlm.nih.gov; Isom et al., 1992). Both auxiliary subunits have an extracellular Ig-like fold, but the $\beta$1 subunit is non-covalently associated with the $\alpha$ subunit (Isom et al., 1994 review; Isom et al., 1996).

When expressed in Xenopus oocytes, the presence of the $\beta$1 subunit modulates the channel gating properties by accelerating the rate of inactivation (ibid). Mutation analysis indicates that neither the intracellular domain nor the glycosylated residues are required for this function (McCormick et al., 1998). However, the Ig-like fold is believed to participate in the presentation of the charged residues of the A/A' strand of $\beta$1 for association with the $\alpha$ subunit (molecular recognition, adhesion, and ligand binding) (McCormick et al., 1998). Also, a 5-fold increase in the number of functional channels being expressed at the cell surface is
correlated with the association of β1 with the αβ2 complex as indicated by the amplitude of peak Na+ current. [McCormick et al., 1998; Isom et al., 1995]. The β2 subunit (SCN2b human chromosome 11, murine chromosome 9) is expressed in central neurons only, and is covalently bound to the α subunit (www.ncbi.nlm.nih.gov; Jones et al., 1996). Its expression is correlated with insertion of the α subunit into the cell membrane [Isom et al., 1995]. Association of β2 with the Na+ channel α subunit also modulates channel gating properties, and causes up to a 4-fold increase in capacitance of transfected Xenopus oocytes by increasing the surface area of plasma membrane microvilli (Isom et al., 1995). This is a unique combination of properties for an ion channel auxiliary subunit, and may indicate a role in localizing Na+ channels in central neurons (Isom et al., 1995; McCormick et al., 1998).

2.9 Role of Sodium Channels in Inter and Intracellular Communication in the Central Nervous System

As described above, the heterotrimeric sodium channel complex in brain consists of the pore-forming α subunit and 2 β subunits. While the β subunits do not contribute to pore formation, they are important functional modulators of channel gating and voltage dependence of activation and inactivation (Isom et al., 1992; Isom et al., 1994; Isom et
The $\beta_1$ subunit effects the translocation of the $\alpha$ subunit to the cell membrane, typical of its role in many sodium channels expressed in other tissues (Isom et al., 1992; Isom et al., 1994). The $\beta_2$ subunit is central nervous-specific, and is believed to guide the $\alpha\beta_1$ complex localizing it at high density to the nodes of Ranvier (Isom et al., 1995). Aspects of sodium channel physiology are discussed in section 2.8.

As with other channel proteins, the sodium channel participates in complex interactions with the extracellular matrix and the intracellular milieu via the $\beta$ subunits. Besides their roles in modulating expression and functional characteristics of the channel, the $\beta$ subunits are believed to be the link in intra and intercellular communication at the nodes of Ranvier and perhaps at synaptic junctions (Malhotra et al., 2000; Xiao et al., 1999).

Immunoglobulin-like (Ig) domains located in the extracellular region of the $\beta$ subunits belongs to the V-set cell adhesion molecule (CAM) superfamily (Isom et al., 1995). This region of $\beta_2$ strongly resembles F3/contactin, which is known to interact with extracellular matrix proteins tenascin-C and tenascin-R (Srinivasan et al., 1998). Tenascin-R has been shown to be involved in neuronal fasciculation and growth cone guidance in vitro (Xiao et al., 1997; Lochter and Schachner, 1993; Pesheva et al., 1993; Taylor et al., 1993; Xiao et al, 1996). It has been postulated that the $\beta$ subunits act as CAM's, and as such, they may
recruit ankyrinG to intracellular binding domains (Davis et al., 1993; Dubreuil et al., 1996; Hortsch et al., 1998; Malhotra et al., 1998). Ankyrins are spectrin binding proteins associated with the cytoplasmic side of the cell membrane of many cell types and have been shown to bind many ion channels (Bennet, 1992). AnkyrinG co-localizes with NrCAM and neurofascin possibly as part of the process of assembling the nodes of Ranvier (Davis et al., 1996). Therefore, it appears that homophilic interactions with tenascin, perhaps between the nascent myelin sheath and the β subunits of SCN2a, produce a signal extracellularly that mediates CAM binding to intracellular domains, thus recruiting ankyrin to the intracellular surface (Xiao et al., 1999). Cell-cell contact then is mediated via sodium channel subunits by providing a communication link between the extracellular matrix and the cytoskeleton (Bartsch et al., 1993; Fuss et al., 1991).

Tenascin-R binding has also been shown to potentiate sodium channels by increasing their probability of opening, increasing single channel conductance and up-regulating existing silent channels (Xiao et al., 1999). It is possible that this increased channel conductance is the signal from tenascin binding to the intracellular environment (Xiao et al., 1999). The clinical significance of proper functional modulation of sodium channels by extracellular proteins and CAM’s is demonstrated by the finding that mutation of a critical cysteine residue in the Ig fold of the
β1 subunit is implicated in familial epilepsy (McCormick et al., 1998). This site is thought to interact with extracellular matrix molecules, thereby altering the channel conductance and causing excitotoxicity (Xiao et al., 1999).

In addition to the role of β subunits as the communication link in cell-cell contact between neurons and glia, the α subunit is phosphorylated by serine/threonine and tyrosine kinases (Levitan, 1999; Cantrell et al., 1996; Hilborn et al., 1998; Ratcliffe et al., 2000). Sodium channels are also phosphorylated directly by PKA, PKC and the cAMP/dopamine pathways (Rossie and Catterall, 1987 & 1989; Cukierman, 1996). Phosphorylation is a ubiquitous method of post-translational modification. It is a rapid, reversible means of regulating expression, localization, and function of ion channels by tying intracellular messenger systems such as G-proteins and the Ca**-activated IP3 cascade to channel excitability and cellular location (Levitan, 1999).

In general, the effects of tyrosine phosphorylation on ion channels depress excitability, while dephosphorylation by phosphatases has an opposite effect. Recently, it was shown that receptor tyrosine kinases (RTK's) responding to growth factor ligands modulate Na+ channel excitability both as an acute response and over the long term (Hilborn et al., 1998). PKA and PKC also inhibit Na+ currents during development
and may also mediate growth factor-induced inhibition (Hilborn et al., 1998). These interactions are thought to have the greatest impact on the developing nervous system. However, there are indications that RTK’s, through the action of src and ras, continue to affect sodium channel function in mature neurons and contribute to neuronal plasticity throughout life (Hilborn et al., 1998; Ratcliffe et al., 2000).

New findings suggest that Na+ channels may be ligands for the extracellular carbonic anhydrase (CA) domain of the receptor protein tyrosine phosphatase, RPTPβ (Ratcliffe et al., 2000). Binding of the CA domain to extracellular matrix proteins and the β1 subunit of the Na+ channel is thought to bring the intracellular phosphatase domain of RPTPβ in contact with tyrosine substrates of the Na+ channel. RPTPβ may also be involved in positioning the Na+ channel at the nodes of Ranvier along with ankyrin (Ratcliffe et al., 2000). Like the RTK’s, RPTPβ is most active in the embryonic stage of development (Hilborn et al., 1998). During the neonatal period, a short, but catalytically active form is still associated with the channel. The catalytically inactive form, phosphacan remains bound through the extracellular CA domain to the β1 subunit of Na+ channels in mature neurons (Ratcliffe et al., 2000). The fact that RPTPβ is primarily expressed in glia, and to a lesser degree
in neurons, suggests that the isoforms bound to neurons may participate in intercellular signaling particularly important during myelin development (Ratcliffe et al., 2000).

Ultimately, the action of reversible phosphorylation by several mechanisms including CAM and growth factor stimulation affects downstream transmission at synaptic junctions directly or indirectly by modulation of ion channels. Depolarization of the synaptic terminal causes the release of neurotransmitters from synaptic vesicles. This produces a change in ionic permeability in the post-synaptic membrane, thus altering the membrane potential in the downstream neuron. Hence, disturbances in phosphorylation of ion channels would be expected to have far reaching consequences functionally and developmentally.

Immune responses are also governed by phosphorylation pathways involving src homology (SH) motifs present in intracellular domains of immune cell surface receptors (Tamir et al., 2000; Rohrschneider et al., 2000). These immunoreceptor tyrosine based inhibitory motifs (ITIM’s) are believed to be docking sites for recruitment of cytoplasmic phosphatases having a src2 homology domain (Malbec et al., 1998; Smith et al., 1998; Maeda, et al., 1999). These surface receptors participate in reversible inhibition of immune receptor activation by src kinases (Malbec et al., 1998; Smith et al., 1998; Maeda, et al., 1999). This process of immune inhibition is key to the attenuation of activation
signals initiated by immunoreceptor tyrosine-based activation motifs (ITAM’s) which are important to cell proliferation in activated immune responses (Daeron et al., 1995; Vely and Vivier, 1997; Yamanashi et al., 2000; Tamir et al., 2000).

Inflammation is the net result of competing activation and inhibition pathways involving recruitment of complement, Ig molecules with ITIM motifs, activated macrophages and mast cells (Sylvestre and Ravetch, 1994; Hazenbos et al., 1996; Sylvestre et al., 1996; Titus et al., 1987). The ratio of ITIM and ITAM sequences present in cell surface receptors and their efficacy at recruiting their catalytically active ligands governs the outcome of the immune response at the site of inflammation (Ravetch and Lanier, 2000). Unchecked inflammation is known to cause overexpression of cytokines, and in some cases, involves loss of proapoptotic signals that are induced as part of immune inhibition (Pearse et al., 1999). Failure to check cytolytic activity of NK (natural killer) cells whose properly functioning class I receptors contain SHP ITIM’s are thought to contribute to graft rejection and the development of autoimmune disease (Long, 1999).

These inhibitory domains are believed to have a broader range of expression on non-hematopoetic cells (Ravetch and Lanier, 2000). ITIM and ITAM activity may be implicated in other activation/inhibition mechanisms such as those governing both acute and long term ion
channel plasticity in the nervous system. Somatic mutation or other events leading to imbalances in these highly intricate signal transduction systems may tip the scales where conditions such as stress, infection, or aging are present.

2.10 Animal Models of Experimental Autoimmune Encephalitis

Postvaccinal encephalitis (PVE) was first observed within a few years following vaccination for rabies introduced by Pasteur (Compston, 1998 overview). This observation was later verified experimentally reproduced in monkeys by Rivers and Schwentker (1935) by immunization with the Semple virus grown in rabbit spinal cord. Control animals immunized with normal spinal cord also developed PVE (Rivers and Schwentker, 1935; McFarlin and MacFarland, 1982a). Both the experimental and control animals displayed perivascular inflammation and demyelination in the CNS indicating that the virus was not responsible for the disease pathology, but rather the spinal cord tissue was encephalitogenic under certain immunization conditions. The experimental form of PVE is now known as experimental autoimmune encephalitis (EAE); this disease has been induced in many strains of several species using a variety of CNS-derived antigens, and is currently the best animal model for the human central nervous demyelinating encephalitis, multiple sclerosis.
The keys to producing EAE are the species and strain (genetic background), choice of autoantigen, mode of sensitization, choice of adjuvant, and age and sex of the host animal (Lassmann, 1983).

**Autoantigen**

An autoantigen is considered 'encephalitogenic' if it stimulates an immune response capable of triggering inflammation (Mcfarlin and MacFarland, 1982a & b; Swanborg, 1988; Tuohy et al., 1998). The hallmark pathology of MS is demyelination typically found in focal lesions of the brain, and more widespread in the spinal cord (Section 2.2). The course of the disease can vary, but is typically chronic with episodic relapses (Section 2.1). Therefore, the myelin proteins, myelin basic protein (MBP) proteolipid protein (PLP) and myelin oligodendrocyte glycoprotein (MOG) have been most intensively studied.

The choice of autoantigen will also dictate where the pathology will develop in the CNS. For example, animals immunized with MBP develop demyelination where the myelin sheath is thickest in the spinal cord or medulla oblongata (Lassmann and Wekerle, 1998 overview). MOG-induced disease is most evident where myelin is thin such as areas of the forebrain and cerebellum (Linnington et al., 1993).
Other CNS antigens have been studied for their ability to induce EAE. These include S100β, and astrocyte-specific protein, and GFAP, glial fibrillary acidic protein, a non-myelin glial protein (Kojima et al, 1994; Berger et al., 1997).

**Mode of Sensitization**

EAE can be induced in virtually all animals either by direct injection of these antigens or by transfer of activated T lymphocytes into syngeneic animals (Paterson, 1960; Ben-Nun et al., 1981). In fact, activated T lymphocytes are essential to the development of EAE. Almost invariably, passive transfer of EAE by T lymphocytes produces an acute, monophasic disease with little or no demyelination or associated tissue damage (*ibid*). Furthermore, it is known that the inflammation observed in MS due, at least in part, to the presence of activated T lymphocytes. T cells, however, do not appear to be responsible for demyelination or other tissue damage, gliosis and axonal degeneration, associated with MS.

Occasionally, a chronic or relapsing progression of disease can be induced with a single injection of T lymphocytes (Mokhtarian et al., 1984). The pathology of the resulting EAE is slight demyelination localized to the perivascular parenchyma of the CNS, and most closely resembles acute disseminated leukoencephalomyelitis (Lassmann and Wekerle, 1998).
Immunizations of animals with whole tissue or purified myelin protein extracts can produce widespread demyelination in most animals. The course of the disease is often chronic progressive or relapsing approximating that of MS (Lassmann, 1983). Immunologically, these animals not only have activated T lymphocytes recruited to areas of inflammation, but there are also abundant activated macrophages and autoantibodies to the experimental antigen (humoral response).

Macrophages clear away cell debris resulting from cytolytic activity of the CD4+ Th1-type T cells with MHC II specificity and are responsible for some of the tissue damage associated with severe forms of EAE (Berger et al., 1997; Section 2.3). The presence of the activated macrophages, and in some instances, activated complement, causes widespread apoptosis of the T cells in the region of inflammation (Pender et al., 1991). In rodents, these T cells predominantly use TCR Vβ8.2 except in the highly susceptible SJL strain, which is deleted for much of the TCR region of the genome. SJL mice are able to substitute Vβ12 for Vβ8.2 (Schmeid et al., 1993; Greer et al., 1996).

The development of a humoral response to the myelin autoantigen correlates with the induction of demyelination and disease progression observed in the animals (Linnington and Lassmann, 1987). Animals with extensive or severe demyelination also have high antibody titers to the autoantigen (ibid).
Adjuvant

Although multiple injections of MBP into some hosts can induce EAE, single injections of MBP in complete Freund's adjuvant are sufficient to induce EAE in a variety of species including mice, rats, guinea pigs and monkeys (Raine et al., 1980; Swanborg, 1988; Wekerle, et al., 1994). Addition of freeze-dried Mycobacterium tuberculosis (strain H37RA) makes the adjuvant complete and by activating the MHC II system, rendering the host response to the immunogen highly effective (Janeway and Travers, 1997; Section 2.3). Specifically, it has been shown that M. tuberculosis upregulates bcl3 which is known to prolong the life of T cells rendering the acute phase immune response more efficient (Mitchell et al., 2001). In addition, the use of adjuvants have an effect on the development of the long term memory pool which is important in mounting later responses to the immunogen (Mitchell et al., 2001).

Injections of Bordetella pertussis (organism or toxin) are required in some strains of mice, to produce EAE from whole spinal cord (Korngold et al., 1986). The toxin acts to permeabilize the blood brain barrier allowing serum proteins and lymphocytes access to the CNS (Harris et al., 1991; Wekerle, 1992).
Age and Sex of the Susceptible Animal

Immunization of neonatal mice induces a T\textsubscript{h}2 type of response, thus protecting the animal for a long time from the encephalitogenic effects of autoantigen challenge (Alvord et al., 1965; Forsthuber et al., 1996; Section 2.2). An analogous situation is observed in MS (Alvord et al., 1965). This is not absolute as it has been shown that rodents normally resistant to EAE induction can develop a severe EAE response to MOG in incomplete Freund's adjuvant (Lassmann and Wekerle, 1998). A similar situation has been observed in marmoset MOG-induced EAE (Genain et al., 1996). Also, females develop autoimmune disease far more frequently than males and therefore, are used almost exclusively for the development of experimental autoimmune disease models.

Species and Strains

As discussed above, it is difficult to induce recurring disease in most animals by T lymphocyte transfer. However, the SJL and PJ mouse strains can develop spontaneous relapsing disease with a single lymphocyte challenge. During the course of the disease, the animals shift their immune response from dominant MBP encephalitogenic epitopes to cryptic determinants of MBP or PLP (Lehmann et al., 1992; Cross et al., 1993). This phenomenon of 'epitope spreading is discussed in detail in Section 2.9. In effect, the spread of autoreactivity to

55
additional new determinants causes a lack of focus of the immune response resulting in an uncontrolled, self-perpetuating immune response.

Chronic disease can be induced in many different animals by multiple challenges as described above (Rivers et al., 1933; Tabira et al., 1984). Lesions in the acute and chronic forms of EAE are the same. The major difference governing the persistence of the disease is the ability of the animal to mount a strong humoral response and produce high affinity antibodies as discussed above (Linington and Lassmann, 1987; Linington et al., 1988). This is often accompanied by complement activity, but is not absolute. The extent of demyelination is the net result of the amount of T cell infiltration and autoantibody expression (Lassmann and Wekerle, 1998). Areas of high numbers of T lymphocytes and low antibody titers show widespread inflammation of CNS tissue, but only slight perivascular demyelination. A strong autoantibody response accompanied by a low number of T lymphocytes leads to discrete focal lesions with extensive demyelination [*ibid*]. The latter situation in conjunction with a chronic, recurring course of disease symptoms most closely approximates MS (Linington et al., 1992).

Interestingly, demyelination does not have to be present for animals suffer disability. Different species (and strains) are sensitive to different MBP epitopes. The resulting disease varies widely from a
monophasic acute course of muscle weakness accompanied by weight loss with no demyelination to complete paralysis and death where the animals have substantial demyelinating lesions in the CNS (Wekerle et al., 1994; Lassman and Wekerle, 1998 overview).

MBP T cell-induced EAE in Lewis rats is an acute, monophasic disease with no demyelination. The rat recovers spontaneously after 18 days (Wekerle et al., 1994). However, MOG-induced EAE in these animals is a chronic relapsing disease characterized by severe demyelination (Johns et al., 1995). Similar findings have been reported for MOG-induced EAE in mice and primates (Amor et al., 1994; Mendel et al., 1995; Genain et al., 1996). It appears that MOG induces both T cell and B cell autoimmune responses, perhaps why disease induction with this component of myelin is so successful.

**EAE vs. MS**

Although some EAE animal models approximate the clinical and pathological features of MS, some groups of MS patients are not represented by any EAE model. One such group of patients is characterized by a high number of T cells and macrophages accompanied by severe demyelination, but no antibody titer or complement activity (Lassmann and Wekerle, 1998). Also, MS lesions can show complete destruction of oligodendrocyte progenitor cells with an absence of remyelination (*ibid*). This situation has not been observed in any current...
EAE animal model. Animal models for direct CD8+ cytotoxicity and/or γ-δ TCR expressing lymphocytes are also absent in current animal models of MS (Lassmann and Wekerle, 1998).

Increasingly, investigators and clinicians are focusing on damage to the nerve axon in MS. This is now known to occur in areas of normal myelination in MS brain tissue (Section 2.1). Virtually all of the current EAE models are of glial and astrocytic proteins and none represents an axonal protein. The study presented in this dissertation presents the first axonal model of EAE using the CNS type 2 voltage-gated sodium channel (SCN2a) as autoantigen.

Although EAE animal studies have revealed much about the pathology of inflammation in CNS tissue, they have contributed to several lines of evidence indicating that inflammation alone will not necessarily result in tissue destruction (Birnbaum and Antel, 1998). Animal models have been fruitful in the development of several new therapies for MS such as oral tolerance using a peptide of MBP, or administration of random peptides (Copaxone). Treatment with β interferons Avonex and Betaseron, and T cell vaccination (Bitar and Whitacre, 1988; Adorini et al., 1992; Johnson and Panitch, 1992; Mitchell, 1993; Paty et al., 1993; Ebers, 1994; Stinissen et al., 1997
review). All of these therapies alone or in combination provide some relief to some patients, but none completely stops or reverses the effects of chronic disease (Stinissen et al., 1997).

2.11 Epitope Spreading

The elusive nature of the trigger that initiates MS has led investigators to consider the early events leading to the disease as distinct from the progression of MS (Martin et al., 1992; Raine, 1997). It is difficult to link an environmental trigger to late onset symptoms because the disease causing agent or conditions are usually gone, and only the ongoing pathological process remains. In this way, damage to tissues caused by some viruses can induce persistent inflammation causing the release of organ-specific peptides into an environment of immuno-reactive cells (Allen and Brankin, 1993; Miller et al., 1997). If any of these newly released peptides is able to bind MHC determinants on an APC either directly or by being ingested by macrophages and then displayed on the cell surface, autoreactivity can occur. In addition, if one of these peptides is recognized by B cell surface Ig, a new autoreactivity is likely to occur (McRae et al., 1995; Yu et al., 1996; Tuohy et al., 1997, 1998; Vanderlugt et al., 1998). This has been demonstrated in several autoimmune diseases where the response to the primary autoantigen
has waned and emerging autoreactivities appear (Vanderlugt et al., 1998). 'Epitope spreading', as this process is called, and has been shown to endogenously prime new autoreactivities that are often unrelated to the initial trigger of the disease (Vanderlugt et al., 1998).

Epitope spreading was dismissed for some time as an 'epiphenomenon' that accompanies disease progression, and several theories as to the cellular mechanism exist (Yu et al., 1996; Tuohy et al., 1997, 1998). It has been suggested that this diversification of autoreactivities is a Th1-linked phenomenon because it is most frequently observed in Th1-mediated diseases like MS and EAE and insulin-dependent diabetes in the NOD mouse (Elson et al., 1995). More recently, investigators working with insulin-dependent diabetes in the NOD mouse have found evidence of Th2 feedback as a possible mechanism of epitope spreading (Tian et al., 1997). A third proposed mechanism for this phenomenon is that antigen-specific B cells, by far the most efficient APC's, may be responsible for diversification of the immune response (Mamula and Janeway, 1993).

In addition, a variety of experiments have demonstrated that the development of new autoreactivities occurs in a specific order of progression, thus presenting a picture of a hierarchical cascade of autoreactivities (Yu et al., 1996). An example of this is Theiler's murine encephalitis virus (TMEV) where the reactivity to the viral antigen is the
primary trigger for the onset of CNS inflammation, but the progression of the disease is characterized by a reproducible cascade of "neo-autoreactivities" in CNS tissue (Miller et al., 1997). Similar observations have been made for relapsing EAE, an experimental model of MS (McRae et al., 1995; Yu et al., 1996), and systemic lupus erythmatosis (SLE), an autoimmune disease directed at nuclear antigens (James et al., 1995; Topfer et al., 1995).

The significance of epitope spreading to the progression of autoimmune diseases like MS is illustrated in a study that examined this phenomenon in two groups of patients with isolated monosymptomatic demyelinating syndrome (IMDS) (Tuohy et al., 1997). One group had the monocentric, monophasic form and the other had the multicentric form of IMDS. The monocentric form of IMDS has a 5-10% risk of progressing to MS (Tuohy et al., 1997). On the other hand, the multicentric form has a 50-90% risk of becoming MS (Tuohy et al., 1997). All patients were evaluated within weeks of onset of symptoms, and were evaluated at 2-month intervals (Tuohy et al., 1997). The result of stimulating the patients peripheral blood mononuclear cells (PBMC's) with an array of overlapping synthetic peptides that span the entire length of the major component of myelin, PLP, indicated that that epitope spreading occurred in both groups of patients (Tuohy et al., 1997). However, the monophasic patients that did not go on to develop MS were able to
sustain their autoreactivities and focus them on a few epitopes of PLP, eventually selecting a core epitope (Tuohy et al., 1997). In the multicentric patients and all of the ones that went on to develop MS, one autoreactivity would fade slowly while new ones emerged (Tuohy et al., 1997). The shift in autoreactivity in these individuals was to non-overlapping PLP epitopes (Tuohy et al., 1997). The neo-autoreactivities could occur abruptly and were also observed in the patients with the greatest activity on MRI (Tuohy et al., 1997).

Although this is an interesting correlation, the most compelling evidence comes from animal models of relapsing EAE where the animals were immunized with TMEV or with an encephalitogenic epitope of PLP (Tuohy et al., 1997, 1998; Miller et al., 1997). In TMEV-induced disease, the initial response was overtaken by myelin epitopes (Miller et al., 1997). In the case of PLP-induced EAE, the relapses coincided with the emergence of a predictable series of neo-autoreactivities to PLP and MBP epitopes (Tuohy et al., 1998). The pathophysiological significance of this is that it was possible in both disease models to halt a relapse and prevent further progression of the disease by administering the next autoreactive epitope in the cascade (Tuohy et al., 1998; Vanderlugt et al., 1998). This means that induction of tolerance autoreactive determinants not involved in the spreading cascade have no effect on the outcome of the disease (Vanderlugt and Miller, 1996; McRae et al., 1995; Yu et al.,
1996). To date, it has not been possible to halt the progression of MS. Therefore, strategies for identifying diversification of autoreactivities in this disease are of great importance and suggest potential for future directions in the development of therapies for MS.
<table>
<thead>
<tr>
<th>Locus Name</th>
<th>Phenotypic Effect</th>
<th>Flanking Chromosomal Markers</th>
<th>Human Syntenic Region</th>
<th>Candidate Genes in this Region</th>
</tr>
</thead>
<tbody>
<tr>
<td>eae1</td>
<td>Incidence</td>
<td>Chromosome 17 6p21</td>
<td></td>
<td>HLA II</td>
</tr>
<tr>
<td>eae2</td>
<td>Incidence</td>
<td>D15Mit51-56 5p13-p15;8q22-23</td>
<td></td>
<td>IL7r, GHR, PrLR, LIFR, C6,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>C7</td>
</tr>
<tr>
<td>eae3</td>
<td>Incidence</td>
<td>D3Mit76-105 3q25-q27;4q28-q33;1p12-p31</td>
<td></td>
<td>FCGR1, IL6R, CSFM</td>
</tr>
<tr>
<td>eae4</td>
<td>Incidence Index</td>
<td>D7Mit69-40 11q13-15;15q11-26</td>
<td></td>
<td>IDDM4, MHC I (minor)</td>
</tr>
<tr>
<td>eae5</td>
<td>Incidence Index</td>
<td>D17Mit10-150 D17105-51 6p21-22.1;19p13.3</td>
<td></td>
<td>Non-HLA</td>
</tr>
<tr>
<td>eae6</td>
<td>Incidence Incidence Severity, Duration</td>
<td>D11Mit16-2 D11 Mit21-11Nds29(IL4) D11Mit2-140 22q12;7p11-13;2p12-23;16p13.5q31-35</td>
<td></td>
<td>Chemokines, cytokines Immunoregulatory loci</td>
</tr>
<tr>
<td>eae7</td>
<td>Incidence Severity</td>
<td>D11Mit36-330 17q11-23</td>
<td></td>
<td>IDD4, ORCH3, NOS2, CNP1, small cytokines</td>
</tr>
<tr>
<td>eae8</td>
<td>Incidence Severity</td>
<td>D2Mit25-200 D2Mit148-200 20q13</td>
<td></td>
<td>CD40</td>
</tr>
<tr>
<td>eae9</td>
<td>Duration</td>
<td>D9Mit22-105 11q22-23;15q21-23</td>
<td></td>
<td>CD3, Thy-1, IGIF, IDD2</td>
</tr>
<tr>
<td>eae10</td>
<td>Onset</td>
<td>Dm114-147 4q21-25;1p25</td>
<td></td>
<td>EGF, CFi, NF-k, PGFr</td>
</tr>
</tbody>
</table>

**Table 2.1.** Genetic Loci Associated with EAE, continued
Table 2.1. continued

Abbreviations used in Table 2.1:
IL-interleukin
GH-growth hormone
C-complement factor
LIF-leukemia inhibitory factor
PrL-prolactin
FCG-high affinity Fc receptor for IgG
IDD-insulin-dependent diabetes
CSFM-macrophages colony stimulating factor
ORCH-orchitis susceptibility gene
EGF-epidermal growth factor
NF-k-light chain gene enhancer
CNP-Cyclic nucleotide phosphodiesterase
NOS-nitric oxide synthase
Idif-IFNγ inducing factor
Cf-complement component factor
PGF-platelet growth factor

www.informatics.jax.org
CHAPTER 3

MATERIALS AND METHODS

3.1 RNA Extraction

The α subunit of the type 2 voltage-gated sodium channel (SCN2a) was chosen as a candidate antigen to base these studies of autoimmunity to axon-specific antigens in the central nervous system. The extracellular segment connecting segments 5 and 6 of Domain I [DI(S5-S6)] of the channel was the first region cloned because it appeared to be relatively hydrophilic and contain pharmacologically important sites. Also, the non-transmembrane portions of Na+ channels cloned to date have shown that these sequences are typically channel-specific and sub-type specific. In other words, an immune response to these non-conserved regions is likely to be directed at that channel subtype alone and not cross react with other conserved transmembrane proteins. This region is 387bp long generating a 127 amino acid protein.

No more than 0.5gm of frozen tissue was processed at a time, or if using cultured cells, $10^7$ cells are the maximum recommended unless a
large scale procedure is necessary. The CNaIIa cell line was used for RNA preparations once it became available (gift from Dr. William Catterall). This cell line has the distinct advantage of being a source of the specific sodium channel, SCN2a that was preferable for conducting these experiments. CNaIIa is a CHO-K1 cell line that is stable transfected with the full length rat SCN2a in the pZEMRVSP6 vector (West et al., 1992). The conditions for culturing these cells are described in section 2.15.

The RNA was extracted in a sterile 13mL plastic tube in TRIZOL (Life Technologies, Rockville, MD) using a Tissuemizer (Tekmar, Cincinnati, OH). Typically 1mL of TRIZOL was sufficient for extraction so long as the tissue or cells occupied no more than 10% of the volume of TRIZOL. The tissue/cells were disrupted at room temperature (RT) with the Tissuemizer until there were no pieces of solid tissue or cell aggregates remaining. The mixture was incubated at RT for 5 minutes, then centrifuged at 5,000 RPM in a Beckman or Sorvall low speed centrifuge to remove any solid particles.

The supernatant was transferred to a 1.5mL microfuge tube, and 0.2mL of CCl₃/mL TRIZOL was added. The tube was shaken vigorously by hand for 15 seconds and incubated for 2-3 minutes at RT. This solution was centrifuged at full speed in a microfuge for 15 minutes at 4°C. The upper, aqueous phase was then transferred to an autoclaved
microfuge tube. The RNA was precipitated with an equal volume of isopropl alcohol and incubated at RT for 10 minutes. The total RNA was pelleted at full speed using a microfuge for 10 minutes at 4°C. The supernatant was carefully removed so as not to disturb the pellet. The pellet was washed with 1 mL 75% ethanol, and gently vortexed until the pellet is in partial suspension. The RNA was pelleted at no more than 7,500 x g in a microfuge for 5 minutes at 4°C. The pellet was partially dried either in the open air at RT or in a speed vacuum dryer under vacuum, but not spinning. The RNA pellet was resuspended in DEPC-H2O or 0.5% (w/v) RNase-free SDS. The pellet was warmed to 55-60°C for 10 minutes to promote dissolution.

The RNA was quantitated at A260 and at A280. When using a manual spectrophotometer, the concentration was calculated using Beer's law using an extinction coefficient of 40µg/mL (Sambrook et al., 1989). The ratio of A260 /A280 is used to determine the relative purity of the RNA. A ratio of 1.8 or greater was considered sufficient purity to use the RNA in the subsequent first strand cDNA synthesis reaction.

An alternative method of RNA extraction was the use of the RNeasy Kit (Qiagen, Valencia, CA). This kit was used according to the manufacturer's instructions except that passaging the cell lysate through a 20 gauge needle 5-10 times was sometimes more effective than the Qiashredder column step to thoroughly lyse the cells. This kit is has
specific requirements for maximum efficiency for different weights of tissue than the TRIZOL procedure, and these specifications must be adhered to.

3.2 First Strand cDNA Synthesis

For the purposes of obtaining a particular DNA sequence of interest as opposed to making an entire cDNA library, 100-200ng of total RNA were used as the template in the first strand cDNA reaction. The RNA, 20U RNase inhibitor (Life Technologies, Rockville, MD) and sufficient sterile H₂O was added such that the final reaction volume will be 50μL, and incubated at 72°C for 2-3 minutes. The mixture was then transferred to wet ice, and 5μL of 10x first strand cDNA buffer, (Life Technologies, Rockville, MD or whatever manufacturer of reverse transcriptase is preferred), a final concentration of 100mM pre-mixed dNTP’s (Apbiotech, Piscataway, NJ), 0.2μg random primers p(dN)₆ (Apbiotech, Piscataway, NJ), and lastly, 5U of AMV reverse transcriptase (Life Technologies, Rockville, MD or APbiotech, Piscataway, NJ). The reaction was incubated at 42°C for 1-2 hours. The resulting RNA/cDNA double-stranded products were then treated with 20ng/μL DNase-free RNase A to remove the RNA leaving only the cDNA templates for use in PCR. At this point, the RNase-treated first strand cDNA can be stored at
-80°C preferably in 5μL single use aliquots. The commercial First Strand cDNA kits were also used for making the first strand cDNA when available (Apbiotech, Piscataway, NJ).

3.3 Polymerase Chain Reaction

PCR primers were designed to the 5 prime sense strand and 3 prime antisense strand of the extracellular region between DI and DII of the rat brain SCN2a as no human sequence was available at that time (Noda et al., 1986a). This region was designated FP1 indicating that it would be the first fusion protein cloned from the Na+ channel for use as an immunogen in mice. Restriction enzyme sites were incorporated into the primer sequence so that the resulting RT-PCR products could ultimately be directionally cloned into the pGEX-2TK vector (Apbiotech, Piscataway, NJ). The position of the restriction sites will also result in the FP1 insert being expressed in-frame once subcloned into the pGEX-2TK vector.

The BamHI site is indicated in bold in the sense primer (34mer), and the EcoRI site is indicated in bold in the antisense primer (35mer) below.

FP1 Sense Primer: ACACGGATCCAAACCTGAGGAATAAATGCTTGCAG
FP1 Antisense Primer: CACGAATTCCGTATGTTTTCCCGGCAGCACGCAA
The primers were custom synthesized by either Oligo's Etc. (Wilsonville, OR) or Life Technologies (Rockville, MD). The concentrated stock solutions were stored at -80°C; working dilutions were stored at -20°C.

Typically 2-4μL of first strand cDNA was used for the primary PCR amplification. Three PCR reactions were set up for each new primer set tested, each with the same buffer, but containing a final concentration of 1.5, 2.5 or 4mM MgCl₂ respectively. The negative control was usually a complete reaction mixture without the template. This was important for each PCR run to ensure the primers had not become contaminated during the course of repeated use. A control primer set was used in a separate set of reactions as a positive control to ensure that the first strand cDNA template and the Taq polymerase were working. The positive control primers most often used were the β-actin primers.

β-Actin Sense Primer: GACTACCTCATGAAGATCCT
β-Actin Anti Sense Primer: CCACATCTGCTGGAAGGTGG

An approximate Tm (melting temperature) could be calculated for all new primers using a value of 2°C for each A or T base, and a value of 4°C for each G or C base. Five degrees Celsius was then subtracted from that value, and used as the annealing temperature in test reactions.

The PCR reactions were done in a PE Biosystems Thermocycler (Foster City, CA) or a DNA Engine PTC-200 (MJ Research, Boston, MA). A typical reaction was done in a total volume of 50μL, although 25μL
reactions were frequently used for direct colony screening. PCR buffers, Taq polymerase and NTP's can be obtained from many sources. At the present time, virtually any source would work for this type of RT-PCR application where the desired PCR product is under 1kb in length, and the primers are sufficiently ‘tailed’ so as not be sensitive to exonuclease activity present in most Taq preparations. A 50μL reaction was assembled as follows: 5μL of the 10x reaction buffer, sufficient autoclaved double distilled H2O such that the final volume will be 50μL, 5μL of 10x stock 2mM dNTP’s (premixed), 30-50ng of each primer, 2.5mM MgCl₂, 10-20ng of first strand cDNA template, and approximately 5U of Taq polymerase. All reaction components were added except the Taq, and gently but thoroughly mixed. Some thermocyclers do not have heated lids, and the reaction needs to be protected from evaporation by layering 35μL of mineral oil on top of each reaction. The reactions were then briefly centrifuged in a microfuge to remove any air bubbles, and to pull any droplets off the sides of the tube.

All PCR reactions were ‘hot started’ meaning that the reactions were loaded into the cycler with the heating block at a temperature greater than 85°C, and held at 94°C while the Taq polymerase was added to each reaction for a minimum of 3 minutes. This fully separates the
DNA strands so that the polymerase and primers have access to the unpaired DNA strands. It is very important to gently mix the Taq into each reaction without disrupting the oil layer.

The cycling conditions varied depending on which stage the template was at. For example, first round PCR using first strand cDNA as a template and the FP1 primers was: hot start, followed by 94°C for 30 seconds, 55°C for 1 minute, 72°C for 1 minute and 30 seconds for 35 cycles. The optimal cycling conditions for reamplifying the FP1 PCR product using the FP1 primers are: hot start, followed by 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds for 35 cycles. A one time final extension step of 72°C for 5 minutes can be added when working from first strand cDNA as a template, but it was not necessary and not used for reamplifications or colony screening PCR. The conditions used for colony screening pGEX or PCR 2.1 TA clones involved vector primers or a vector-FP1 primer combination. The conditions were: hot start, followed by 94°C for 30 seconds, 50°C for 1 minute, 72°C for 1 minute for 35 cycles. The vector primers were either M13 forward or reverse and the corresponding FP1 primer. The MgCl₂ concentration used for vector-FP1 primer combinations was 2.5mM. The conditions for the β-actin positive control primers were: hot start at 95°C followed by
95°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute for 30-35 cycles. These primers are conserved in human and mouse. The optimal concentration of MgCl₂ for this primer set was 2mM.

3.4 Cloning and Colony Screening

The most effective way to 'capture' PCR products for further amplification and purification is to use a TA' vector such as PCR 2.1 (Invitrogen, Carlsbad, CA). TA vectors capitalize on the fact that one or more adenosines are added to the 3 prime ends of the DNA strands during the PCR. These vectors are sold precut and tailed with thymidine so that the PCR products need only be added to the vector, annealed and ligated. Ligation reactions were done in a 10μL total volume, adding 1μL 10x reaction buffer (Roche Molecular Biochemicals, Indianapolis, IN), 4μL H₂O, 2μL PCR 2.1 vector, 2μL PCR products, and 1μL T4 DNA ligase (Roche Molecular Biochemicals, Indianapolis, IN). The contents of the reactions were mixed thoroughly and gently centrifuged in a microfuge. The reaction was then incubated overnight in the cold room in a 14°C water bath. The ligated RT-PCR products were transfected into the DH5α bacterial host purchased as subcloning competent cells according to the manufacturer's instructions (Life Technologies, Rockville, MD). Only 1-5μL of the ligation reaction was used per 50μL of competent cells.
as the efficiency of transfection is highly sensitive to the concentration of DNA (Sambrook et al., 1989). The transfectants were plated on LB agar plates containing 50μg/mL ampicillin (Sigma, St. Louis, MO) in aliquots of 50-200μL/plate. The plates were inverted and grown overnight at 37°C.

The following day, colonies were transferred to 'master plates' using sterile toothpicks or pipette tips. The master plates were LB agar containing 50μL/mL ampicillin, and had a grid printed on the bottom of the plate so that each colony could be assigned coordinates. The master plates were grown overnight at 37°C, and the following day, 25-30 individual colonies were screened by direct colony PCR under the conditions described in the previous section. The colony serves as the DNA template. A pipette tip is touched to the colony and then dipped into the PCR reaction and swirled gently.

The products of the colony PCR were then viewed on a 0.8% (w/v) agarose gel in TBE buffer (0.09M Tris base, 0.09M boric acid, 0.001M EDTA, pH8) containing 5μL of 10mg/mL ethidium bromide to see which ones contained an insert of the correct size (Sambrook et al., 1989). Usually, 8μL of product were sufficient to see on a gel and 1μL of a 10x loading buffer (10 mM EDTA, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 50% (v/v) sterile glycerol) was needed to add density to the sample. The dye front makes it easy to follow the progress.
of the run. This will vary if the desired product is small, perhaps under 100bp. Alternatively, small PCR products can be seen more readily if the gel is stained in a 5µg/mL bath of ethidium bromide for 5-10 minutes. The gel can be destained in H2O if necessary.

Different combinations of primers can be used for colony screening since the PCR 2.1 vector has PCR primer sites, a sense vector primer and antisense specific (FP1) primer were used to generate a fragment of approximately 300bp. It is important to use at least one vector primer/specific primer combination at some point because very tiny amounts of contaminating primers or DNA from the RT-PCR and ligation reactions can lead to false positive results. The primers used were the M13 reverse primer and the T7 promoter primer each in combination with an FP1 primer. This is also the reason that the initial colonies must be transferred to master plates to minimize the carry over of contaminating DNA from previous reactions. The FP1 primers used for the colony screening were a shorter version of the ones listed above. In other words, a set of FP1 primers was made having the same Na+ channel sequences, but without the restriction site and tail sequences. These were used in colony screening reactions.

The colonies thought to contain the correct insert were picked from the master plate, grown as 5mL overnight liquid cultures in LB containing 100µg/mL ampicillin at 37°C with shaking in a Lab-Line
incubator-shaker (Barnstead-Thermolyne, Dubuque, IA). In the morning, 0.5mL of each culture was transferred to a sterile 1.5mL freezing vial (Sarstedt, Newton, NC) and 0.5mL of a freezing solution consisting of a 1:1 mixture of sterile glycerol and LB broth was added to the culture aliquot. The colony pick stocks were then stored at -80°C. It is important to note that any culture to be used for Sequenase sequencing should not be grown in an enriched broth such as YT-G.

3.5 Sequencing

The remaining 3-5mL of overnight cultures of the colonies identified as having an insert of the correct size were then used for sequencing. The cells were lysed and denatured according to the standard 3-step procedure including a final RNase treatment described in the First Strand cDNA Section (Sambrook et al., 1989). The concentration of the DNA can be measured by running 1μg of the DNA from each clone on a 0.8% (w/v) agarose gel as described and comparing it to a standard of known concentration. Alternatively, the concentration can be determined spectrophotometrically by measuring the A260 and calculating the concentration using an extinction coefficient of 50μg/mL. The Gene Quant spectrophotometer calculates the concentration automatically (Apbiotech, Piscataway, NJ).
The DNA template must be denatured prior to sequencing. Most
DNA sequencing was done using the Sequenase 2.0 system (USB,
Cleveland, OH), so the method for denaturation required 1-2μg of good
quality DNA, in 0.2N NaOH and 0.2mM EDTA in a total of 50μg.
Incubate at 37°C for 30 minutes. Neutralize with 1/10 volume of 3M
sodium acetate, pH 5.2. Extract the denatured DNA by adding an equal
volume of a 1:1 mixture of equilibrated phenol and chloroform
(Sambrook et al., 1989) by shaking vigorously, then centrifuging for 2
minutes at RT. Recover the aqueous phase and put in a clean Eppendorf
tube. Re-extract the organic phase by adding an equal volume of H₂O,
shake, centrifuge, and then add the re-extracted aqueous phase to first
aqueous phase. It is important to remove any EDTA prior to the
sequencing reaction because EDTA inhibits Sequenase polymerase.

Precipitate the denatured DNA by adding 2.5 volumes of 95%
ethanol (or absolute ethanol if available) and store at -25°C for 30
minutes. Centrifuge the solution for 15 minutes at RT or 4°C, and
remove the liquid from the pellet. Wash the pellet adding 0.5mL cold
70% ethanol, shaking the tube or resuspending the pellet with a pipette.
Centrifuge for 5 minutes at RT, and carefully remove the liquid. Air dry
or speed-vacuum dry the pellet. Resuspend the dried pellet in 10μg
dH₂O. At this point, 1μg of the denatured DNA can be run on an agarose
gel, and a broad but distinct band should be visible.
The sequencing reaction is assembled EXACTLY according to the manufacturer's instructions. The primers used for the reaction can vary. For the purpose of screening TA clones, the vector primer flanking either side of the insert can be used. The Sequenase sequencing system is based on the dideoxy chain-termination method (Sanger et al., 1977). In these reactions, α[^32P-dCTP] was used to label the DNA fragments (Apbiotech, Piscataway, NJ). The sequencing products are then loaded on to a gel containing 1x TBE buffer, 8M ultrapure urea (USB, Cleveland, OH), 5.7% (w/v) polyacrylamide, 0.3% (w/v) bis-acrylamide and 0.5% ammonium persulfate in a final volume of 100 mL. The solution is filtered through a 0.2μm filter to remove undissolved particles and other impurities. Just prior to pouring the gel, 30μL of TEMED is added to catalyze the polymerization of the polyacrylamide matrix. The volume of the sample loaded onto the gel depends on the type of comb used. If a lot of samples are processed at once which is the case with colony screening, a shark's toothed comb is used allowing 2μL sample volumes. This preliminary screen of several clones is generally done with one vector primer, and if there are enough slots for the samples, two loadings can be done, staggered by about 60% of the length of the gel to allow more sequence to be read for each clone. The electrophoresis conditions vary depending on the size of the fragment to be sequenced, but typically the gel is run at 1800 V for several hours.
When feasible, the three best clones are selected from the primary sequencing, and sequenced fully in both directions making sure that the restriction sites in the FP1 primers were intact and in the translational frame of the gene.

3.6 Subcloning FP1 into pGEX-2TK

The clones with the best sequence match were then regrown overnight as 1mL cultures in LB with 100µg ampicillin from the master plate. Alternatively, a master plate colony or the frozen stock can be streaked out, grown over night and a single colony picked from the streak. The DNA was extracted as described in the previous section. Approximately 1µg was double-digested with EcoRI and BamHI using the manufacturer's 10x reaction buffers. A few microliters of the reaction was loaded onto an agarose gel to check that the digestion had gone to completion meaning that 2 bands, one the size of the vector (4.9kb) and one approximately 400bp were seen. Any undigested DNA will transfec the new host efficiently, thus, making it time consuming to find the correct transfectants. Once the digestion has been verified, the reaction can be stored at −25°C.

The pGEX-2TK expression vector is sold as a vial of 25µg of DNA. Therefore, the vector needs to be transfected into the recommended host,
XL-1 Blue (Stratagene, San Diego, CA), and propagated in culture. The DNA is extracted as previously described. The extracted vector DNA is also double-digested with EcoRI and BamHI and a single band of 4.9kb should seen on the gel. Both the double-digested pGEX vector and the FP1 insert released from the PCR 2.1 vector can be purified by band isolation by various methods.

One effective method for generating extremely clean quantitative amounts of DNA is the DEAE paper method of band isolation. DEAE-NA45 paper strips were purchased from Schleicher & Schuell (Keene, NH), and activated before use by rinsing with 10mM EDTA, pH 7.6, for 10 minutes at RT, then rinsing in 0.5N NaOH for 5 minutes at RT. Rinse the strip several times in sterile dH₂O and stored submerged in sterile dH₂O for a few weeks. This increases the binding capacity of the paper. The digested DNA was run in a preparative well until the individual fragments were clearly separated. The progress of the gel can be viewed with long wave UV (quantitative setting). A strip of the DEAE paper is then cut to match the width of the well. A slit is cut through the gel on the anodal edge of the band of interest. The gel continued to run preferably at about 100 V for several minutes. The disappearance of the band cannot be checked with the DEAE paper in place because it will cause crosslinking of the DNA to the paper.
At this point, the paper strip was removed from the slit and transferred to a tube containing NET wash buffer to remove residual agarose (20mM Tris, pH 7.6 and 5mM EDTA). The strip was rinsed briefly, then transferred to an Eppendorf containing 250μL high salt NET buffer (20mM Tris, pH 7.6, 5mM EDTA, 1.5M NaCl). The strip must be submerged. It can be cut in two, and split between two tubes if necessary. The strip in the high salt NET was then incubated at 68°C for 10-45 minutes swirling the tube now and then. For DNA fragments larger than 1kb, increase the temperature to 80°C.

The high salt NET buffer was transferred to a clean tube and 50μL of clean high salt NET added to the paper strip to rinse it. This was added to the tube containing the larger volume of high salt NET. The ethidium bromide and loading dye can be removed from the DNA by serial extractions with an equal volume of water-saturated n-butanol but it is not necessary. The tube was shaken well to mix the phases, centrifuged briefly and the aqueous (bottom) phase recovered. The DNA was precipitated with 2.5 volumes of 95% ethanol at -20°C for at least 5 hours. The DNA was pelleted in a microfuge for 20 minutes at 4°C. Once the ethanol was removed, the pellet was usually invisible. The DNA was resuspended in H₂O and re-precipitated to remove the NaCl by adding 1/10 the volume of 3M sodium acetate and 2.5 volumes of 95% ethanol.
The XL-1 Blue bacterial host is the recommended strain for transfection and expression of the pGEX-2TK vector (Stratagene, San Diego, CA). The FP1 insert was ligated into the double-digested pGEX vector as described in Section 3.4 or with a ligation kit (5 prime-3 prime, Boulder, CO). The transfection was done according to the manufacturer's instructions. It is important to note that the manufacturer recommends YT-G or LB broth for propagation of this host.

The medium used in the agar plates did not affect growth, but all liquid propagation (except for preparation of sequencing templates) was done in YT-G as the yield is better (Sambrook et al., 1989).

The plating and colony screening was done as previously described except that the vector primers for the pGEX vector are a different sequence than the PCR 2.1 primers. A large number of positive clones were obtained at this point. A few representative ones were selected and only the 5 prime and 3 prime ends were sequenced to verify that the frame of the fusion construct was intact.

3.7 Expression of FP1-GST Fusion Protein in pGEX-2TK

The pGEX series of vectors use the lac promotor to drive expression of the glutathione-s-transferase marker gene and the downstream cloned insert, thus generating a GST-fusion protein. The
GST 'tag' is approximately 26kDa, and is not expressed in mammals. The FP1 portion of the fusion protein is 15.5kDa, making an approximately 42kDa band on SDS-PAGE. When injected into mice, GST has not been shown to produce any ill effects except a dermatitis condition in some strains of mice (Apbiotech, Piscataway, NJ).

Expression from the lac promotor using 0.1mM IPTG is highly efficient and detection of the GST fusion tag is simple using the commercially available goat anti-GST on western blots. The disadvantage of bacterial expression systems is the tendency to sequester fusion proteins in inclusion bodies rendering the fusion protein difficult to solubilize (Coligan et al., 1995).

Clones selected from the colony screen were then cultured in YT-G as 1-2 mL cultures for approximately 6 hours at 37°C until the A600 was 0.5-2.0. This indicated that the bacteria were in the log phase of growth, the optimal time to induce expression of the FP1-GST fusion protein. Inductions were done at 37°C with shaking by adding 0.1mM IPTG and continuing to incubate for 1 hour. One hundred microliter aliquots of each culture were analyzed by SDS-PAGE by adding an equal volume of 2x SDS sample buffer consisting of 0.125M Tris, pH 6.8, 10% (w/v) SDS, 10% (v/v) MSH, 20% glycerol, 0.004% (w/v) bromophenol blue (Laemmli, 1970). The samples were boiled for 3 minutes then sonicated for 30 seconds on ice in bursts a few seconds to destroy the DNA. Depending
on the comb used, 5–10μL of each sample was loaded onto a 12.5% (w/v) SDS polyacrylamide minigel and separated for approximately 120 Volt hours or until the dye front has reached the bottom of the gel (Sambrook et al., 1989). A protein size marker was always run simultaneously. The Rainbow markers are especially good because proteins of known size are visible during the separation (Apbiotech, Piscataway, NJ). Typically, half of this gel would be directly stained in Coomassie Brilliant Blue R-250 and the other half would be electroblotted (Sambrook et al., 1989).

3.8 Western Blotting

The gel was then transferred onto nitrocellulose or preferably onto Optitran BA-S83 reinforced nitrocellulose membrane (Schleicher & Schuell, Keene, NH). Optitran has the added advantage of greater mechanical stability than nitrocellulose so it can be stripped and reprobed up to 4 times. The Optitran membrane was soaked in H₂O before use (10% methanol for nitrocellulose). Equilibrate the gel in transfer buffer for 10 minutes at RT. All pieces of filter paper and sponges must be soaked in transfer buffer before assembly. Otherwise, they will pucker and trap air bubbles making the transfer uneven.

The transfer procedure can be done in a variety of ways depending upon the type of electroblotter used. In all cases, it is essential to have
the blotting membrane on the anodal side of the gel because the proteins will be transferred in the anodal direction. A dry blot system was used successfully for small and mid-sized proteins up to about 60kDa (Dr. Kottil Rammohan personal communication). This consists of two flat carbon electrodes about 14x14cm. The bottom plate is the anode so the proteins are transferred downwards. Three layers of thick filter paper (Whatman 3mm) was soaked in Lower buffer (20% methanol) and laid firmly on the bottom carbon platform (anode). Three layers of filter paper soaked in Middle buffer (20% methanol) were then placed on this. The pre-wet blotting membrane was layered on top of these. The gel equilibrated in Middle buffer was placed on top of the blotting membrane and rolled out using a glass pipette to gently expel any air bubbles. A layer of filter paper soaked in Middle buffer was put on this, and finally 3 layers of filter paper soaked in Upper buffer (8-amino-n-caproic acid, 20% methanol). Transfer is complete in approximately 120 V/hours.

Alternatively, a Genie semi-dry blotter (Idea Scientific, Minneapolis, MN) was used where the entire assembly of filter paper, blotting membrane and the gel is immersed in a single buffer consisting of 10% methanol, 0.2% (w/v) SDS, 25mM Tris, 200mM glycine, pH 8.2 (Towbin et al., 1979). The assembly is then squeezed into a tight chamber with a front and back plate. If using the platinum plates, the
front plate is the anode. Like the dry blotter, this worked well for small
to mid-sized proteins. Transfer was complete in 1-2 hours at 125
V/hour.

For larger proteins, the conventional wet blotting tank (BioRad,
Torrey Pines, CA, and APbiotech, Piscataway, NJ) was the most effective.
The buffer composition and gel/blot assembly was the same as for the
semi-dry blotter, but the transfer was done overnight for 220 V/hours at
4°C. It is important to have a magnetic stirrer in the bottom of the tank
to circulate the buffer.

In the process of taking apart the gel/blot 'sandwich', it is
advantageous to have the gel side up so that the gel can be outlined in
ballpoint pen and the origin and marker lane designated. Also, the gel
can be peeled back slowly to be sure all the Rainbow marker bands have
transferred. A fine line of ballpoint was made through the center of each
marker protein and its color indicated with a letter (e.g. b for blue). This
allows for no ambiguity when comparing the probed gel to the
autoradiograph later.

The Optitran blot was rinsed thoroughly in H₂O at the end of the
electroblot procedure to remove residual buffer salts and SDS. At this
point, the membrane can be air dried and stored between filter paper at
RT or placed in a Tupperware container or blotting bag in 1x TBS (20mM
Tris, 200mM NaCl, pH 7.6) containing sodium azide to prevent contamination and placed at 4°C (Harlow and Lane, 1988). Ideally, the blot should be probed within 24 hours.

Prior to probing the blot it must be blocked for at least 1 hour. Blocking times of a few hours can be done at RT, but longer times, overnight blocking must be done at 4°C to avoid contamination. There are a few different blocking solutions that can be used, but the most effective one is 'Blotto'. Blotto is made of 5% (w/v) non-fat dry milk and 1%(w/v) bovine serum albumin (BSA) in TBS. The blots were probed with the primary antibody diluted in a 1:5 dilution of Blotto over night at 4°C on a rocking platform. The blot was often blocked for about 20 minutes with a 1:100 dilution of normal serum from the host of the secondary antibody. This could be done at any time prior to probing the blot with the secondary antibody. Secondary antibodies raised in goats tend to have fewer background problems (Dr. Daniel Coover personal communication) and were chosen when available. The names of the antibodies, vendors and typical dilutions used for probing western blots are shown in Table A.1.

The secondary antibody was diluted according to the manufacturer's recommendations in TBS or TBS-T depending on the amount of background. This needed to be determined empirically for each antibody.
The majority of blots were developed using the ECL blotting system, which is a chemiluminescent detection system (Apbiotech, Piscataway, NJ). It is important to follow the manufacturer's instructions for the chemiluminescent kits because the reagents need to mixed proportionately and handled in a timely manner. The standard ECL and ECL Plus kits were both used. ECL Plus stabilizes the chemiluminescence so that longer development times can be done which is an advantage for weaker signals.

Autoradiography of the chemiluminescent blots was done with 2 different films. Hyperfilm MP has a broad range of sensitivity and is recommended for both isotopic and chemiluminescent applications (Apbiotech, Piscataway, NJ). BioMax MR has an advantage in that it has an ultraclear background, and light signals can be interpreted more easily (Apbiotech, Piscataway, NJ). BioMax is a 'sided' film and it is important to put the emulsion side (dull side) against the blot, otherwise it will not be exposed.

3.9 Purification of FP1-GST Fusion Protein for Immunizations

The FP1 fusion protein turned out to be extremely insoluble. It appears that part of this extracellular region fold back into the
membrane and therefore, this represents a significantly hydrophobic domain of the protein (Catterall, 1993). Also, there was every indication that the fusion protein was being sequestered in inclusion bodies.

A variety of approaches were tried including boiling the induced bacterial culture in 8M urea, and then trying to titrate the denaturant (Sambrook et al., 1989). Every effort was made to avoid strong detergents such as SDS because it would not be compatible with the immunization procedure. Also, because the fusion protein was so insoluble, it was not possible to use commercially available approaches to purifying the protein such as the Glutathione Sepharose 4B resin. This resin can be used under conditions of up to 3M urea, but not higher urea concentrations (Apbiotech, Piscataway, NJ). The lowest concentration of urea where appreciable amounts of FP1-GST were released from the bacteria was 5M.

A method developed for the extraction of proteins from inclusion bodies was adapted using 4M guanidine hydrochloride (Coligan et al., 1995). Guanidine is a stronger denaturant than urea, and therefore, can be used at lower concentrations.

3.9.1 Lysis of the Amplified FP1-GST/XL-1 Bacterial Cultures

Following a 1hour induction of the XL-1 host transfected with FP1-pGEX by addition of 0.1mM IPTG at 37°C for 1 hour, the cultured cells
were pelleted and lysed as follows. The cells were resuspended in Lysis buffer containing 100mM Tris-Cl, pH 7, 5mM EDTA, 5mM DTT, 5mM benzamidine-HCl. This buffer must be made fresh prior to use. The EDTA and the benzamidine in this buffer are present as protease inhibitors and were substituted with a protease inhibitor (pi) 'cocktail' that contains a broad range of protease inhibitors and is available in pill form. This is very simple and effective (Life Technologies, Rockville, MD). To this was added 200µg/mL lysozyme to help remove the peptidoglycans from the outer cell wall. A volume of 4mL Lysis buffer/gram wet weight of cells was recommended. The cells were vigorously suspended in the Lysis solution using a homogenizer (Tekmar, Cincinnati, OH). Complete cell lysis is very important to the success of this procedure, and the published protocol suggests using a French Press for this purpose. There wasn't one available to me, so I passaged the cell suspension through ascending gauge needles until the cells would no longer pass. Then the suspension was sonicated on ice until it was no longer a viscous solution. It is important to sonicate in bursts of a few seconds. The lysed cells were pelleted at 22,000 x g for 1 hour at 4°C to clarify the solution.

Washing

Unbroken cells and inclusion bodies were pelleted. The supernatant was decanted and an aliquot was run on SDS-PAGE to be
sure that the protein of interest is not soluble under these conditions.
FP1-GST was not in the supernatant fraction, so the inclusion body
pellets were washed in Wash buffer: 100mM Tris-Cl, pH 7, pi pill, 5mM
DTT, 4M urea, 2% (w/v) Triton X-100 (20g/L). This buffer must also be
made just prior to use. Lower concentrations of urea and detergent can
be used, but it had already been determined that FP1 was not soluble
under these conditions. A volume of 10mL/gram of cells was used, and
the pellet was resuspended by homogenization. The mixture was
centrifuged at 22,000 x g for 30 minutes at 4°C, and the supernatant
discarded. This step was repeated using 6 mL/gram of cells. This step
was repeated twice more centrifuging for 20 minutes each time. The
supernatant should be perfectly clear. If not, the washing was repeated.
The final pellet was resuspended in 4-6mL/gram of cells of Wash buffer
without urea or Triton. Centrifuge at 22,000 x g for 30 minutes at 4°C;
this step removes excess urea and detergent. The washed pellets can be
stored at -80°C at this stage without any deleterious effects.

3.9.2 Extraction of FP1-GST from the Cell Lysate
The washed inclusion body pellet was resuspended in 4mL/gram
original weight of cells in Extraction buffer containing (50mM Tris-Cl, pH
7, pi pill, 4M guanidine-HCl, 5mM DTT) using a homogenizer. Up to 8M
guanidine-HCl can be used to fully extract all remaining proteins.
Centrifuge the suspension at 100,000 x g for 1 hour at 4°C. Carefully decant the supernatant and filter it through a 0.2μm sterile syringe filter. This step removes any large cell wall debris that remained insoluble until now.

3.10 Sample Preparation for SDS-PAGE

A small aliquot of the extracted protein can be analyzed by SDS-PAGE by precipitating the protein prior to loading it on the gel. This was done by taking a 25μL aliquot of protein in the guanidine Extraction buffer and adding 225μL cold (0°C) 90% ethanol. Mix well by inverting the tube, and chill to -20°C for 10 minutes. Pellet the protein in a microfuge for 5 minutes at full speed at 4°C. Carefully remove the supernatant and suspend the pellet in 250μL cold 90% ethanol and vortex. Centrifuge as before and resuspend the final pellet in 25μL of SDS sample buffer. Boil the sample 3 minutes before loading on the gel. Depending on the intended use of the purified protein, the next step may vary (Coligan et al., 1995). In the case of FP1-GST, the extracted pellet suspension was dialyzed overnight at 4°C against 1000 volumes of PBS.

Removal of the guanidine denaturant from the solution causes FP1 to flocculate out of solution. This step was necessary because guanidine is toxic to the animals. The suspension was centrifuged in a microfuge
at full speed for 10 minutes at 4°C. The flocculate was resuspended in a small volume (approximately 1mL) of sterile PBS. A few microliters was then put in an equal volume of SDS sample buffer, boiled and run on a gel to assess the purity and approximate the concentration. Due to solubility problems of FP1-GST, it was quantitated on a gel by comparing it to a known protein standard using SDS-PAGE. Other soluble proteins were quantitated using the Bradford bicinchonic acid method (BCA) by making a standard concentration curve with a commercial albumin solution, then making several dilutions of the protein of unknown concentration (Pierce Chemicals, Rockford, IL; Sambrook et al., 1989). After a 30-minute incubation at 50°C or overnight at RT, the standard and unknown reactions were measured at A562. The results were calculated using the linear regression mode or manually on linear graph paper (Dr. Daniel Coovert personal communication). It was this material that was prepared as the immunogen for the animals.

3.11 Preparation of FP1-GST in Adjuvant for Immunizations

The purified fusion protein was resuspended at a concentration of 4mg/mL in complete Freund’s adjuvant (CFA) containing 200μg/mL Mycobacterium tuberculosis (H37RA strain) and used as a stock solution (Life Technologies, Rockville, MD). The stock solution of FP1/CFA was
diluted with incomplete Freund's adjuvant to make a series of different dosages of the fusion protein and H37RA. Groups of 5 mice from 2 different strains were immunized with increasingly dilute concentrations of FP1 and adjuvant. In this way, the effect of the FP1 immunogen and the *M. tuberculosis* dosage was titrated. This was done as a preliminary study to determine the optimum dose of the experimental antigen and the adjuvant. Tables 3.1 and 3.2 summarize the immunization groups of both the BALB/c and SJL/J strains of mice. An unimmunized control group of 5 mice from each strain was subjected to the same behavioral testing and weight monitoring as the immunized groups of mice.

Each dose of FP1/CFA was administered as an emulsion made by transferring the oily mixture of adjuvant and suspended fusion protein back and forth between 2 glass 5mL syringes through a 3-way stopcock (Fisher Scientific, Pittsburgh, PA). This step is crucial to the success of the immunization because the emulsion must be the consistency of peanut butter to immobilize the *M. tuberculosis* and fusion protein. This allows the immune system access to the autoantigen over a period of weeks. A stiff white paste results from the vigorous movement of the oil between the syringes until the plunger can hardly move.
3.12 Immunization Regimen

Two strains of mice were used for the preliminary experiments to see which strain(s) would develop the experimental encephalitis. Sixty female 6-week old BALB/c and SJL/J mice were purchased from Harlan Sprague-Dawley Labs (Indianapolis, IN). Initial weights and limb strength testing was done. The animals were sheared across the lower back with a full sized electric animal clipper (gift from Dr. Kate Meurs) a couple of days prior to immunization. There were 10 groups of 5 mice in each strain that received a different dose of FP1/CFA, and the remaining mice were the uninjected control group.

The immunizations were administered intradermally on the back of the animal above the hind quarters using a 1 or 2mL glass syringe with 0.01mL calibrations and a 18 gauge needle (Fisher Scientific, Pittsburgh, PA). This region is easy to access, and makes the immunogen available to the dendritic system beneath the skin. This area is rich in antigen presenting cells (APC's) which are very effective at mounting the immune response for sensitization. The inguinal lymph nodes in the groin would be harvested and cultured if T cell studies or passive transfer were to be done. The animal was positioned on a wire cage top so that it will naturally grasp the wire. Firm pressure was applied to the base of tail with the thumb and forefinger of one hand so that the animal's grasp
tightened while being injected. The other fingers of the same hand were pressing against the neck to help restrain the mouse. It is crucial to keep an eye on the mouse’s breathing because it is easy to shut off the airway with this kind of hold. The first immunization consisted of a total volume of 100μL/site. Generally, 2 sites were injected each time. This time point was designated Day 0.

On Day 2, 200ng of Pertussis toxin resuspended in sterile H₂O was administered intravenously (retro-orbital route) under isofluorane anesthesia. The isofluorane was made up as 15% (v/v) in light mineral oil. A gauze pad was taped to the inside lid of a glass jar. A small amount of the isofluorane was poured onto the pad and the mouse was put in the jar with lid on for 1 minute (Dr. Emily Chen personal communication). This anesthetized the mouse for no more than a minute, so speed was important. The mouse was held in one hand with the little finger tightly around the base of the tail, and the thumb and index fingers grasping the scruff of the neck such that if the mouse woke up, it couldn’t bite. The needle is guided along the nose next to the eye and inserted carefully between the eyeball and the tear duct (Dr. Kottil Rammohan personal communication). All Pertussis injections were done with a standard disposable 1mL tuberculin syringe fitted with 26 gauge needles. The isofluorane gauze pad needs to be changed after every 5 mice. On Day 14, the animals were given a second identical dose of the
immunogen except that it was administered in incomplete Freund's adjuvant (no H37RA supplementation), and 150ng Pertussis toxin boost was given intraperitoneally Day 14.

The histology and behavioral testing of the unimmunized control mice provided a baseline against which the experimental, immunized mice were compared. Serum was obtained from immunized and control mice at time points greater than Day 21 either by retro-orbital test bleeding or by collecting the blood from the abdominal cavity at the time of perfusion. The blood was diluted 1/5 with PBS and incubated overnight at RT. The blood was centrifuged in a microfuge at 3,500 RPM (1000 x g) for 10 minutes to remove the red and white cells. The serum (top phase) was collected and stored at –80°C. A pool of sera was made to use as a primary antibody for probing western blots.

An optimum dose of 200µg FP1/50µg H37RA was chosen from the preliminary dose groups. The immunizations were done the same way, but an additional control group was added. This consisted of 10 mice immunized with the 200µg induced GST vector lysate and no FP1 insert in 50µg CFA. This was done to ensure that the pathology of the mice was due to the autoantigen, FP1 and not to the fusion tag, the adjuvant or some other contaminant in the protein preparation.

The serum pool from some groups of FP1-immunized mice was adsorbed with an acetone powder preparation of the uninduced FP1-GST
fusion construct in the host DH5α. The acetone powder was prepared as follows: 1gm of cell culture was resuspended in 1-2mL PBS in a 50mL screw cap tube. This was vortex mixed well, and placed on ice. Eight volumes of ice-cold acetone was added, and shaken well. The mixture was incubated on ice for 15-30 minutes, then centrifuged at 4,000 RPM. The supernatant was discarded and the acetone resuspension was repeated at RT. The material was pelleted as before, and this time the cell pellet was spread out on foil to dry. The dry, crumbly acetone powder was added to the primary antibody to a concentration of 1%(w/v). Incubate at RT for 15-30 minutes, then pellet the powder at maximum speed in a microfuge for 1 minute.

3.13 Behavioral Testing

The animals were evaluated on a weekly basis for body weight, walking patterns, balance and co-ordination, forelimb and hind limb strength, and righting reflex. The walking box consisted of a small shoebox with a slit at one end approximately 3cm wide and 8cm high. Into this was slotted a 40cm long (approximately 2 feet long) walkway that looked like a flat-bottomed U in cross section. The walkway was lined with a strip of cash register tape cut to exactly fit the width and length of the walkway. The feet of the mice were inked immediately prior
to walking using a red ink pad for the fore feet and black ink for the hind feet (Behrman et al., 1992). The ink was applied with a long, wood-handled Q-tip. The ink used was standard, non-toxic water-soluble ink, and did not harm the animals. The open end of the walkway where the mouse began the walk test was always placed over a sink so that if the animal jumped or was able to turn and run, it would not escape.

Fore limb strength was evaluated by allowing the animal to grasp the bars of the cage top, while they were being pulled backwards by the base of the tail. Hind limb strength was assessed by hanging the mouse by its hind feet on the edge of the cage so it faced the side of the cage. The ability of the mouse to hold on or pull itself up onto the edge of the cage was determined. Another way of evaluating hind and fore limb strength was to allow the mouse to climb a ladder (Dr. Michelle Basso personal communication). The ladder used for this purpose was a bird ladder purchased from a local pet store.

The righting reflex was determined by holding the mouse supine by the base of the tail and the scruff of the neck approximately 8cm above the bench covered with a foam pad. The mouse was released from the hold at once and it was noted whether the mouse landed on 'all fours' or on its side or back. Each mouse was given two tries at this exercise at the weekly evaluations.
Balance and co-ordination were assessed in a few different ways. The climbing test takes into account elements of balance and co-ordination, but the rot-a-rod test is very sensitive to this capability (Rogers et al., 1996; www.mgu.har.mrc.ac.uk/mutabase/shirpa). A commercially manufactured rot-a-rod was not available to me, so I used a smooth finished cylindrical (not faceted) pencil. This proved to be an excellent tool for testing balance and co-ordination, and to some degree, limb strength. The mouse was placed on the rod (pencil) while the pencil was kept horizontal. The scoring system for the behavioral tests is detailed in Appendix B. Blank data sheets used for recording and organizing the clinical and histological scores for each mouse are found in Appendix C.

3.14 Histopathology

Animals were sacrificed at weekly intervals using a CO$_2$ chamber. The animals were perfused through the heart after cutting the hepatic vein with PBS or Ringer's solution. The perfusate was administered by IV into the left ventricle of the heart (at the apex of the heart). Approximately 20mL of perfusate were delivered over a 10-minute period of time at RT. The brain was dissected and in some cases was immersed into Formalin (12% formaldehyde) for histology using Hemotoxilin and
Eosin (H & E) staining. Hemotoxilin (Gill's Formula; Sigma Chemicals, St. Louis, MO) stains nuclei blue and Eosin stains the cytoplasm a bright pink. The fixed brain tissue was processed by Histotechniques (Powell, OH) as 5-8μm horizontal ‘thick’ sections. Although there was no evidence behaviorally that the animals had pathology in the spinal cord or peripheral nerves, the spinal cord and the cauda equina were dissected in some animals, and examined histologically.

### 3.15 Immunohistochemistry

In many instances, the brain was dissected following perfusion, and then flash frozen in dry ice-cooled isopentane (2-methyl butane) until bubbles could no longer be seen rising from the tissue (Dr. Kottil Rammohan personal communication). The brain was then transferred to a clean plastic scintillation vial stored on dry ice, then transferred to a −80°C freezer in the laboratory. These unfixed, frozen brains were sectioned as 8-10μm horizontal ‘thick’ sections using an IEC Minitome (Needham Heights, MA).

The cryostat chamber temperature was set to −13 to −10°C. The temperature varied largely due to problems with the thermostat. In general, the section will tend to fracture or persistently crinkle up if the temperature is too low. A practical way around this is to press your
thumb against the tissue block for approximately 1 second, then cut the section. This seems to warm up the surface of the tissue, and the sections turn out very clean. The sections were stained with a variety of antibodies to determine whether inflammation was present and what cells were involved in the inflammatory process. All sections were fixed in 95% ethanol for 2-3 minutes, then rinsed 3 times for 2 minutes in PBS prior to addition of the first antibody.

At this point, 50μL of 20% (v/v) sterile glycerol can be pipetted onto the section and a coverslip placed on top. These sections can be stored at 4°C for a few weeks (Dr. Kottil Rammohan personal communication). To remove the coverslip, float it off by placing the slide in a shallow dish of PBS so that the slide is just submerged. Gently lift the coverslip with forceps to loosen it from the slide.

Table A.2 in Appendix A lists the primary and secondary antibodies used for the immunohistochemistry experiments. For some of the antisera, only one vendor was found, but for some antisera, the vendor had been specifically recommended by other investigators in the field (Dr. Richard Wardrop personal communication). Particular attention was paid to the source and dilution of the secondary antibodies (Dr. Daniel Coovert and Dr. Thanh Le personal communication). All primary antibodies were diluted in TBS-T (TBS supplemented with 0.1% (v/v)
TWEEN-20) unless using the Vector MOM kit, then the kit procedure was followed (Vector Labs, Burlingame, CA). All washes were done in TBS-T with 0.2% (v/v) TWEEN-20.

All secondary antibodies were horseradish peroxidase-conjugated (HRP), diluted in either TBS or TBS-T depending on the amount of background observed with a particular antibody. The HRP was developed with the Vectastain AEC (3-amino-9-ethylcarbazole) kit that stains an orange-red that is distinct from the hemotoxilin counterstain (Vector Labs, Burlingame, CA). In situations where the primary antibodies were raised in mouse, e.g. mouse monoclonal antibody CD3*, the Vector MOM (mouse on mouse) kit was used EXACTLY according to the manufacturer’s specifications. It was found that the MOM Block solution was far more effective at a 1:1 dilution in PBS supplemented with 5% normal serum from the host of the secondary antibody (Vector Labs, Burlingame, CA). Also, many of the slides processed with antibodies for immunohistochemistry were counterstained with Hemotoxilin by a method supplied by Vector Labs. This allowed the areas of positive staining to be localized to specific regions or structures in the brain. Spleens from immunized mice acted as positive controls for antibody staining.
3.16 Immunofluorescence of the CNalla/CHO Cell Line

It is very difficult to find a positive control for antibody staining of central nervous voltage-gated sodium channels. The cell line CNalla is a stable transfectant of the full length rat SCN2a gene in the pZEMRVSP6 vector in Chinese Hamster Ovary (CHO) cells (generous gift from Dr. William Catterall). Expression of the SCN2a construct is under the control of the metallothionein promoter that is Zn++ inducible (West et al., 1992). The cells were cultured in DMEM with 5% fetal bovine serum (FBS), 1% (v/v) pen-strep and 1% (v/v) glutamine supplementation at 5% CO₂.

Passaging was done in 1x Versene consisting of 0.53mM EDTA in PBS for 5 minutes at RT (Life Technologies, Rockville, MD). The cells come off the dish very easily when the dish is tapped on the side of the bench. The CHO-K1 parent cell line was obtained from ATCC (Manassas, VA; www.atcc.org) and was grown in Kaighn's modification of Ham's F12 medium due to a requirement for proline, and supplemented with 1% (v/v) pen-strep and 2mM glutamine (Life Technologies, Rockville, MD).

This cell line served as a negative control for western blots of CNalla extracts and whole cell immunofluorescent staining using the pooled sera from immunized and control mice. Finally, membrane
preparations of induced and uninduced CNall a cells were run on SDS-PAGE and western blotted to see if the sera from the immunized and control mice would recognize the full length channel (Figure 4.5).

The CNall a cell membranes were prepared by removing the cells from the dish in Versene and resuspending the pellet in a hypotonic lysis buffer containing 0.3M sucrose, 10mM HEPES pH 7.4-7.6, 10mM KCl, 0.5mEDTA (or pill) and 0.5mM DTT. This buffer should be assembled fresh and 1 mL used to resuspend the cell pellet on ice. The suspension was then put through approximately 4 cycles of freeze-thawing by placing the suspension at -80°C until frozen (a minute or two), then transferring to ice, then to a 37°C water bath. The cells were then passaged through descending size syringe needles from 18-25 gauge. The lysate was spun at 500 RPM for 5 minutes at 4°C, and the pellet was retained. The supernatant was transferred to a clean tube and spun again as before. This pellet was also recovered and the pellets were pooled and resuspended in 50-100μL of SDS sample buffer, boiled and run on SDS-PAGE.

For the purposes of immunofluorescent staining, the cells were passaged and diluted 1:1 in DMEM/FBS after passaging. A few drops of resuspended cells were placed on 1% (w/v) gelatin-coated coverslips that were immersed in DMDM/FBS in the wells of a six well microtiter plate. The cells were allowed to adhere to the coverslip for about 1 hour.
SCN2a expression was inducible with 75μM ZnCl₂ added to the medium for 2-4 hours. Overnight induction can be toxic to the cells. The cells were then washed in PBS and blotted dry around the edges.

The coverslips were placed in a porcelain coverslip holder and immersed in a 1:1 methanol:acetone mixture for 5-10 minutes at RT to fix the cells. The coverslips were then gently blotted. After this fixation step, it is a good idea to put a letter or number on the backside of each cover slip so that they are not accidently flipped over. The cells were rehydrated with PBS for 5 minutes at RT and blocked in 5x Block (5% (v/v) horse serum, 5% (v/v) FBS and 1% (w/v) BSA in PBS) for 1 hour at RT. The primary antibody was applied in 1x Block and incubated for 2 hours at RT. The coverslips were washed 3-6 times for 5 minutes each in PBS containing 1% (v/v) TWEEN-20 if the background is high. The FITC-conjugated second antibody was applied in 1x Block for 40 minutes at RT in a darkened chamber. The coverslips were washed 6 times in PBS with or without TWEEN-20 depending on the background situation. To preserve the fluorescence, approximately 20μL of Vectashield anti-fade solution diluted 1:3 in TBS was added to the coverslips and another coverslip was carefully laid over the top being careful not to trap bubbles (Vector Laboratories, Burlingame, CA). The cells were viewed with a Nikon EC 800 scope.
CHAPTER 4

RESULTS

4.1 RT-PCR of the FP1 Transcript from the CNaIIa Cell Line

The FP1 region of the type 2 voltage-gated sodium channel (SCN2a) was cloned by RT-PCR using total RNA isolated from the CNaIIa cell line (gift from Dr. William Catterall). This is a CHO-K1 cell line (www.atcc.org) that has been stably transfected with the full-length rat SCN2a gene in the pZEM vector (West et al, 1992). The gene is under the control of the SP6 promoter and protein expression is directed from the Zn++-inducible metallothionein promoter (West et al, 1992).

The FP1 primers were optimized to encompass the less well-conserved region connecting S5 and S6 of Domain I in SCN2a (Section 2.7, Figure 2.1). This was also selected as a region of physiological significance since it contains toxin binding sites that are pharmacological hallmarks of sodium channels (Hille, 1992). A BamH1 site was
incorporated in the +3 frame of the sense primer and an EcoR1 site was engineered into the antisense primer to be compatible with the pGEX-2TK vector polycloning site (Apbiotech, Piscataway, NJ).

The first strand cDNA was made using the Pharmacia first strand cDNA kit according to the manufacturer's instructions and primed with pdN6 (Apbiotech, Piscataway, NJ. The double stranded FP1 PCR product was obtained in the first round of amplification with the FP1 primers. The exact fragment size is 386bp, and a band of approximately 400bp was observed on a 1% (w/v) agarose gel (not shown). One microliter of the first round PCR product was reamplified to increase the yield of the FP1 PCR product (Figure 4.1). This re-amplified FP1 PCR product was ligated directly into the PCR 2.1 TA vector for sequencing (Invitrogen, SanDiego, CA).

4.2 Sequencing the FP1 cDNA TA Clone

The PCR product was then sequenced in both directions using the T7 and M13 reverse vector primers using the Sequenase 2.0 kit (Apbiotech, Piscataway, NJ). Six base pair substitutions were found (Figure 4.2). Only one of the base pair changes resulted in an amino acid substitution relative to the rat SCN2a template (Figure 4.3). This was a change from Val 311 to Met. The five other base pair changes were
'silent' meaning they occurred in the wobble position, which is a degenerate position in the base pair triplet coding for most amino acids. The FP1 region of the rat SCN2a protein is 99% homologous to the human protein sequence (www.ncbi.nlm.nih.gov). The murine SCN2a has now been cloned (www.celera.com). Serendipitously, the mouse protein has a methionine at position 311, making it identical to the FP1 cDNA.

4.3 Subcloning FP1 into the pGEX-2TK Expression Vector

The DNA from the FP1-TA clone was digested with BamH1 and EcoR1, and the insert was ‘band isolated’ from an agarose gel as described. This insert was subcloned into a BamH1-EcoR1 digested pGEX-2TK vector and transfected into the XL-1 host (Stratagene, San Diego, CA). After master plating a minimum of 100 clones, approximately 25 clones were selected and the colony picks were amplified with both vector and specific PCR primers to be sure they contained the insert (not shown). Two of these colonies were selected for induction of FP1 expression from the lac promoter with 0.1mM IPTG for 1 hour during the log phase of growth (Sambrook et al., 1989).

Several attempts were made to extract the 41.5kDa FP1 fusion protein into the supernatant fraction following cell lysis methods recommended by the manufacturer (Apbiotech, Piscataway, NJ). These
consisted of low molarity urea buffers that failed to solubilize the fusion protein at all. Strong detergents such as SDS were avoided as they are difficult to dissociate from proteins, and are not favorable for the kind of immunizations intended for the FP1-GST immunogen (Harlow and Lane, 1988). Urea concentrations of 5M and greater along with sonication of the cell pellet released the fusion protein from the pellet fraction during cell lysis. Only in 8M urea with repeated sonication was the fusion protein fully extracted from the cell pellet. Unfortunately, other proteins were co-extracted by this method and 80% enrichment of the FP1-GST fusion protein was the best that could be achieved with this method (not shown). This was the material used for the Preliminary Dosage immunizations summarized in Tables 3.1 and 3.2.

Regardless of the method of purification of FP1-GST, the immunogen was suspended in PBS at 4mg/mL. The dried granular Mycobacterium tuberculosis (strain H37RA) was suspended in a separate tube of Freund's adjuvant making this adjuvant complete. The importance of the M. tuberculosis is that it stimulates the MHC I system of antigen presentation (Janeway and Travers, 1997). Specifically, M. tuberculosis upregulates bcl3, a protein that has been shown to stabilize T_{H1} cells thus making the immune response particularly efficient (Mitchell et al., 2001). The aqueous and oily suspensions were mixed together in a series of proportions to result in the immunization dosages.
in complete Freund’s adjuvant listed in Tables 3.1 and 3.2. The final emulsion was readied for injection by rapid, repeated transfer back and forth between glass syringes through a 3-way stopcock as described. This immobilizes the proteins in a stiff emulsion so that they remain available to the immune system at the injection site for a prolonged period of time. The injections were done intradermally where the dendritic antigen presenting cells are abundant and across the lower back, and area that is accessible for repeat injections (Janeway and Travers, 1997). This site is considered comparable to the footpad which is no longer an approved animal protocol.

4.4 Preliminary Immunizations of FP1-GST in BALB/c and SJL/J Mice

Fifty 10-week-old female animals of the BALB/c and SJL/J strains were each immunized in groups of 5 with the preliminary dosages of FP1 and *M. tuberculosis* shown in Tables 3.1 and 3.2. Two additional animals of each strain served as unimmunized normal controls (Harlan Sprague-Dawley, Indianapolis, IN). The schedule of immunizations (dose regimen) was done as described in section 3.11. In addition to the FP1
immunizations on Day 0 and Day 14, this preliminary group of mice received two 200ng intraperitoneal doses of Pertussis toxin on Day 2 and 14 (List Laboratories, Campbell, CA).

All animals were monitored daily for 2 weeks, then weekly thereafter. During the 2-week period of close monitoring, the mice were weighed, and their hind limb strength evaluate by hanging them by their back legs on the edge of the cage or steep-sided bowl. The ability of the mouse to hang on with the hind limbs, and jump down or pull itself up onto the rim was a qualitative measure of hind limb strength. The uninjected mice accomplished this task easily and repeatedly. Mice experiencing hind limb weakness were unable to pull their own body weight up onto the rim of the bowl. Mice with very weak hind limbs slid off the rim and into the bowl without the ability to break their fall.

Fore limb strength was assessed by holding the animal by the base of the tail and placing the mouse on a wire cage top. The animals will naturally grasp the wires with their forepaws. Firm pressure was exerted pulling back at the base of the tail. Normally, an animal can hold onto the wires with sustained firm pressure. Affected animals were either able to resist moderate pulling, or their paws slid easily off the wires with slight pressure.

The immunized BALB/c mice showed little or no deviation from the control mice in that they had sufficient hind limb strength to hang onto
the rim for a period of time, and often pulled themselves out and walked around the rim. Also, little if any diminution of fore limb strength was observed in these mice. The mice groomed well throughout the duration of the experiment, and no significant weight loss was observed. No significant histological changes were noted in these mice (Table 3.1). These observations are consistent with attempts to induce MBP-EAE in BALB/c mice indicating that this strain is relatively resistant to the encephalitogenic effects of the sodium channel and MBP antigens (Tuohy et al., 1988). Some BALB/c mice in certain MBP dose groups went on to develop chronic, later stage symptoms, but even this effect was substrain-specific (Tuohy et al., 1988; Wekerle et al., 1994). One mouse from each dosage group was maintained for observation for 6 months. No change in the condition of these mice was observed.

The SJL/J strain is quite different from the BALB/c strain. This strain has large deletions and rearrangements in the T cell receptor region of the genome (www.informatics.jax.org). It is a strain frequently used for developing the encephalitic response with a variety of experimental antigens (Wekerle et al., 1994). The SJL/J mice tended to be more nervous and hyperactive than the BALB/c strain, and they were noticeably affected by the injections. Their normal circular running motion was slowed or eliminated, and they did not gain weight relative to controls for several weeks following immunization. After about 4 weeks
following the initial injections, the SJL mice appeared to improve. Most of the mice gained some weight, and regained some of their hyperactivity. This kind of acute phase response is typical of SJL mice with the MBP autoantigen and very similar to what was observed with the FP1 immunogen (Tuohy et al., 1988; Wekerle et al., 1994 review). It was noted over a period of a couple of months that some of the SJL mice failed to retain their weight gain. Some became so weak that they were sacrificed. Others died overnight, and it was not possible to obtain tissue for histology because the brain decomposes within hours after death.

A couple of animals from each dose group were sacrificed at consecutive time points in order to follow the histopathology of the induced disease. Prior to perfusion, approximately 0.5mL of whole blood was taken after severing the hepatic vein under deep anesthesia (CO$_2$). Animals whose tissue was only to be used for histological staining then underwent fixation perfusion with 20mL PBS followed by 20mL 4% (w/v) paraformaldehyde/0.1M sodium phosphate, pH 7.4. The fixed brains and spinal columns were transferred to vials of Formalin. This tissue was paraffin embedded and sectioned in 5 micron thick sections, and stained with Hemotoxilin and Eosin (H & E) which provides a high contrast double staining of the nuclei and cytoplasm (Histotechniques, Powell, OH).
Where indicated "histology+" in Table 3.2, the animals in these dosage groups exhibited aggregates of mononuclear cells. The infiltration of mononuclear cells in blood vessels look like small doughnut rings (vascular cuffs). Another common site for infiltration to occur is beneath the meninges, the membrane covering the brain (not shown). This was also observed in several animals, indicating that inflammation was present in submeningeal areas (not shown). Although H & E are not myelin-specific stains, areas of significant demyelination would appear as an area of low intensity staining with these stains. There was no evidence demyelination observed in any of the tissues and no abnormalities of any kind in the spinal cord. These Preliminary Dose animals were treated as a preliminary study to see if inflammation could be induced with the FP1 immunogen, and therefore, full characterization of inflammation in the tissues by immunohistochemistry was not done.

4.5 Western Blot Analysis of the FP1-GST Fusion Protein and the CNaIIa Cell Line

The whole blood collected from both strains at the time of perfusion was centrifuged and some of the sera were pooled and used for immunofluorescence of CNaIIa whole cells and also for probing western
blots of CNaIIa extracts. The uninjected control mouse serum was stored separately and served as a negative control in western blot analysis.

The sera from the BALB/c mice were also stored and aliquots pooled even though these mice were relatively asymptomatic. The individual BALB/c sera were diluted 1/5000 and uniformly show strong reactivity to a band at 42kDa on blots of gels with the induced FP1-pGEX clones (not shown). The pooled sera were diluted 1/500 and adsorbed with an acetone powder preparation of IPTG-induced pGEX vector (no insert) in the DH5α host as described in Materials and Methods section 3.10. The same procedure was followed for sera pooled from immunized SJL/J mice. This pre-adsorbed serum was then diluted to a final 1/1000 dilution and used to probe a western blot of the FP1-GST antigen preparation used to immunize the mice (Figure 4.4). This band corresponds exactly to the band that is positive with the anti-GST control sera in the bottom panel of Figure 4.5 (Apbiotech, Piscataway, NJ). Also, the antibodies developed in BALB/c and SJL/J mice directed towards FP1 can recognize the full length SCN2a expressed in this cell line by western analysis as shown in the top panel of Figure 4.5. This indicates that the animals are capable of developing an autoreactivity to SCN2a in vivo. A comprehensive table of the antibodies used for western blotting is found in Appendix A.
4.6 Immunofluorescence of the CNallα Cell Line

Immunofluorescence of the Zn ++-induced CNallα cell line stained with a 1/500 dilution of pooled sera from immunized BALB/c mice shows a bright halo of fluorescence surrounding the individual cells (Figure 4.6). The sera from uninjected control mice do not stain the induced CNallα cells verifying that the SJL/J mice responded to the FP1 autoantigen specifically, although they did not develop notable pathology. The uninduced CNallα cells do not show reactivity with sera from immunized SJL/J mice (not shown). Identical results were obtained from the BALB/c mice, but are not shown.

4.7 Scale-Up of Selected FP1/CFA Immunizations

A method for lysing cells and purifying only the inclusion body fraction was used to increase the level of purity the FP1-GST immunogen (Coligan et al., 1995). The adaptation of this method is described in Methods section 3.8. The isolated inclusion bodies containing the FP1-GST fusion protein were lysed with 4M guanidine-HCl to ensure complete release of the fusion protein. Guanidine is toxic to animals and was removed by dialyzing the inclusion body lysate against 1000 volumes of PBS causing the fusion protein to flocculate out of solution. The soft
precipitate was the material that was suspended in PBS for use as the immunogen for the mice. Figure 4.7 shows a series of dilutions of the inclusion body purified FP1-GST run on SDS-PAGE and stained with Coomassie Brilliant Blue R-250. The FP1-GST immunogen prepared by this method is approximately 95% pure at the lower concentrations used for the scaled-up immunizations (200μg and 100μg FP1), and about 90% pure for the 1mg high dose immunization group.

The results from the Preliminary Dosage studies did not specify a single optimum dosage of FP1/CFA, but the higher concentrations of FP1 (200 and 100μg) and M. tuberculosis (50 and 100μg) were clearly more effective at producing inflammation in SJL/J mice. Therefore, Three groups of 30 SJL/J mice were each immunized with one of the three selected dosages of FP1/CFA: 1mg FP1/20μg CFA; 200μg FP1/20μg CFA; 100μg FP1/100μg CFA. The FP1/CFA immunizations were done on Day 0 and the boost was done on Day 7. A 200ng Pertussis toxin intravenous injection was done on Day 2, and a 150ng intraperitoneal injection on Day 9 finished the regimen. The boost immunization consisted of 50μg FP1/IFA for all dosage groups.

Occasionally, an animal died or was unable to participate in the behavioral testing leaving 28 animals in the 100μg group and 25 each in the 200μg and 1mg groups. A total of 29 uninjected control mice and 19 "background control" mice immunized with 200μg of a total protein
lysate of the induced pGEX vector with no insert grown in the DH5α host/20µg CFA. The "background control" boost immunization consisted of 50µg of the vector-host proteins/IFA. These mice also received Pertussis on the same schedule as the FP1-immunized mice.

4.8 Behavioral Testing

Weight loss, grooming, handling response, hyperactivity and the presence of whiskers are general indicators of the overall health of the animals. These characteristics and individual behavioral tests were assessed for all mice on a weekly basis. It should be noted that the SJL/J mice were hyperactive at the outset and many of the mice displayed "circling" behavior on a normal basis. In some animal behavior models, development of repetitive circling is a pathological finding, but in this case it was a normal attribute. The scoring system used for individual behavioral tests and attributes is located in Appendix B.

The unimmunized control animals showed a steady weight gain over a period of 4 weeks (Figure 4.8). The vector-host immunized control animals failed to gain weight for the first week following immunization, but caught up after an initial response to immunization. The FP1-immunized animals lost weight dramatically during the first week, and on average, the FP1-immunized animals did not attain the weights of the
unimmunized controls. Thirty percent of the FP1-immunized animals lost weight, and some these animals lost up to 30% of their body weight. This is an extreme amount of weight loss, as those animals became too weak to eat or drink. In situations of severe weight loss, Petri dishes of powdered food were put onto the cage floor, and were slightly wetted to keep the animals from starving or becoming dehydrated. A couple of animals in the 1mg and 100µg dose groups needed to be rehydrated with intraperitoneal saline injections.

A battery of tests was adapted from the SHIRPA website (Rogers et al., 1996; www.mgu.har.mrc.ac.uk/mutabase/shirpa). The SHIRPA site was developed by the Medical Research Council in the U.K. to evaluate mouse radiation mutants. The tests cover a wide range of activities that evaluate balance, strength, and reflexes. Also, other investigators (Dr. Michelle Basso personal communication) suggested additional tests such as the ladder climbing test. Organization and collation of the behavioral data was also a significant task. Appendix C contains a set of tables used to collect the gait evaluation data, weekly behavioral testing scores and histopathology data.

4.8.1 Gait Evaluation

The animals were all subjected to walking tests prior to immunization and at weekly intervals thereafter. This was done by
inking the front paws red, and the rear paws black and placing the mouse in a narrow high-sided walkway that was open at the starting point and fed into a dark box containing food at the end (Figures 4.9 and 4.10; Behrman et al., 1994). Animals that were not grooming well could be spotted easily because they were covered with black and pinkish-red ink that they had not properly washed off. This was typical of animals immunized with FP1 whether they developed other pathology or not. This was not specific to dose group, but rather associated with increasing lethargy and poor motor test results and, in some cases, blindness.

The animals' gait was analyzed for stride length which, for these purposes measured the distance between the middle of the hind foot pad to the same foot pad directly ahead (Figure 4.9; Behrman et al., 1994). The individual stride lengths were averaged over the number of strides counted for each walk. A minimum of 5 strides were counted on each side for each walk. This was done for both the right and left stride lengths to see if the animals had a symmetric stride in the forward direction. Differences of up to 2mm from one side to the other in stride length was observed in all animals and therefore, considered a normal range of stride length variation. Differences of up to 4mm variation in stride length from one side to the other were observed in the vector-
immunized and FP1-immunized animals. This was considered a background level of variation and was included in the number of animals having a stride within an unaffected range of variation.

The unimmunized controls had symmetric, even strides that did not differ by more than 2mm during a walk. Only 20% of the unimmunized mice showed this slight stride length variation and it was therefore, considered a normal variation.

One half of the vector-immunized control mice had a stride length that was different by 2-4mm. This is considered a background (due to acute effects of immunization) level of variation in gait symmetry in this study as it occurred in a control group did not associate with histopathology. Therefore, the black bars indicating normal stride variation in the Gait Analysis histogram in Figure 4.11 include the unimmunized and vector-immunized gait variation. Only one mouse in this vector-immunized control group had a stride length asymmetry of greater than 4mm. This degree of stride length asymmetry was considered abnormal as it was only observed in FP1-immunized mice and associated with histopathology in those animals. The one vector-immunized animal with the abnormal gait was also extremely hyperactive, and never acclimated to handling or the walkway. This is also the animal that fell off the edge of the counter indicating a lack of

123
“visual cliff” response. It is also possible that this animal experienced sensory abnormalities that gave it these behaviors that were otherwise not observed in the vector-immunized mice.

The 100μg FP1 dose group had 58% of mice that showed an abnormal stride length variation. The 200μg FP1 dose group had 36% of animals with asymmetric stride length of greater than 4mm, and 68% of the 1mg dose group of animals had an abnormal stride length. The lightly shaded bars in the Gait Analysis histogram in Figure 4.11 represent the number of animals with an abnormal gait.

Stride width was also measured. Figure 4.9 shows how the distance between the center of one hind foot pad was measured across the stride and perpendicular to a line drawn parallel from the center of the opposite hind foot pad (Behrman et al., 1994). This tends to be constant except under conditions where the animal is very agitated or where sensory and/or motor functions are significantly affected.

Stride width variation was not observed in any control animals, but was observed in all FP1 dosage groups and comprised a small subset of the animals with stride length abnormality. The most severely affected animals dragged a hind foot or had completely erratic strides and were considered ataxic. An animal with assymetric stride length and stride width variations was considered an ataxic animal.
Asymmetric stride length occurred with greater frequency than variations in stride width and levels of stride length variation could vary week to week. Therefore, the number of animals in the abnormal stride group on the histogram are those that had abnormal stride length and possibly width, but did not necessarily retain an abnormal stride until they were sacrificed. Occasionally, this pattern would vacillate back and forth week to week with some animals returning to a gait within normal range of variation. In contrast, animals that developed variations in stride width seldom returned to a constant stride width. Hence, and variation in stride width was abnormal. Animals with abnormal stride width usually had the postural abnormalities described below.

Paw placement was noted, but not quantitated (Behrman et al., 1994; Basso et al., 1996). Typically, the placement of the front paw relative to the rear paw is consistent throughout the walk. However, some mice displayed a loss of coordination of the front and rear paws. Sometimes this occurred bilaterally, but often, only one side was affected and only in FP1-immunized mice. It could vary from walk to walk (week to week), but would usually recur at a later date.

A few mice respond to the narrow walkway erratically by running down the walkway, stopping, turning, not proceeding or even jumping straight into the air. These behaviors were noted, but not incorporated
into the quantitative evaluation of the animal’s gait or clinical score because they were not associated with any other tested behaviors or histopathology.

Several FP1-immunized animals developed a severely hunched posture that gave them variable length of individual strides on a given side that looked like a combination of short hops and long jumps. These mice usually had an abnormal stride length as well (Barlow, 1996). This posture abnormality is indicative of “torso instability” also seen in mice that waddled severely (Crawly and Paylor, 1996). Generally, these mice had several gait abnormalities including uncoordinated placement of fore and hind paws on one or both sides of the body and abnormal stride width. Not surprisingly, these ataxic animals usually had poor limb strength and poor rod test results. Development of an abnormal gait was a good indicator of disability and is present only in the FP1-immunized mice with only one exception in the vector control group.

4.8.2 Hind Limb Strength

The individual test scores are listed in Appendix B. A reduction in hind limb strength that signified abnormality was defined as a hind limb test score of 0-1 for each mouse. This means that the animal can only hang by its hind legs from the rim of the cage before falling into the cage (score of 1) or cannot even hang by its hind legs, and just slides into the
cage (score of 0). All unimmunized mice were able to hang by their hind legs, and pull themselves onto the rim (score of 2) where some mice would sit walk around the rim of the cage. This normal level of hind limb strength is represented by the black bars in the Hind Limb Strength histogram in Figure 4.11. Abnormal hind limb strength (scores of 0-1) are represented by the lightly shaded bars. Some variation was observed from week to week, but an animal that reached a score of 0 usually did not regain a normal score of 2.

4.8.3 Fore Limb Strength

Fore limb strength was considered abnormal if the animal was barely able to grasp the wire cage top while being tugged by the base of the tail (score of 1) or unable to grasp the wire cage top and provide any resistance at all (score of 0). A normal level of fore limb strength (score of 2) was observed by mice that could grasp the wire cage top and could resist being pulled off the wire by several firm tugs at the base of the tail. Results for this test were similar to those found for hind limb strength as shown in Figure 4.11.

4.8.4 Ladder Climbing

Ladder climbing is an innate behavior meaning that all the mice were able to climb to the top of the ladder and reverse direction at the top
of the ladder if it was inverted as a baseline of normal (pre-immunized) behavior. All animals were given 2 chances to climb the ladder at each evaluation. Some animals from both vector-immunized and FP1-immunized groups showed diminished (score of 1) or loss of ladder climbing skills (score of 0) that were both considered abnormal. For some animals, this may have occurred as a result of severe weight loss and muscle weakness during the first 3 weeks post-immunization. Animals that were partially able to climb the ladder were sometimes able to regain the ability to climb the entire ladder and reorient themselves (normal score of 2).

There was a tendency for some animals to become distracted during this test and spend time exploring the ladder instead of climbing the entire length. These animals usually had normal levels of strength and activity and sometimes were not given a score for that week and re-tested the following week. Animals that lost all ability to climb usually remained in the abnormal group (score of 0-1). Results from the ladder climbing tests are shown in Figure 4.11.

4.8.5 Stationary Rod

The stationary rod test complex because it combines elements of co-ordination, balance and strength. Sensory abnormalities were not evaluated in this study, so it is unknown whether or how frequently
these may have contributed to performance of the stationary rod test. It was noted, however, that blind animals did not do well on this test. If an animal performed well on the rod (score of 3-4), this was equivalent to normal behavior indicated by the black bars in Figure 4.11. Many animals performed moderately on the rod (score of 2) from time to time, and usually regained a score of 3-4, so these animals were included in the normal, unaffected group.

Animals that did poorly on the stationary rod (score of 0-1) are included in the affected group for this test. In most instances, these animals also had gait abnormalities, poor hind limb strength, inability to climb the ladder and cerebellar histopathology of grade 2-3 or greater.

### 4.8.6 Torso Curl and Tail Curl

The loss of the ability to bend the torso and touch nose to tail when held upside down was observed in all animals, regardless of immunization status. The development of climb clasping (or grasping) was observed simultaneously along with the loss of torso curl ability in an age-dependent manner. This is known to be a feature of the SJL/J strain due to a mutation in fasciclin, a gene involved in neuromuscular function.
Evidence tail paralysis was slight to absent in all animals regardless of immunization status. This is understandable considering that fact other spinal cord functions also remained normal and no spinal cord pathology was observed.

4.9 Clinical Evaluation

A scale was needed to rank the overall condition of the mice at different time points. This was adapted from an existing neurological scale (Smith et al., 2000). The gait evaluation data and the other behavioral testing data were merged using this system of scoring the various features of each mouse.

Clinical Assessment Scale

0: No discernable reduction in strength, activity level, or behavioral test scores.

1: Some limb weakness (hind or fore limb score of 1).

2: Increased muscle weakness (score of 0-1) and/or diminished ladder climbing ability (score of 1).

3. Inability to remain balanced on a stationary rod (score of 0-1) and/or gait abnormalities (Section 4.8.1); also, may no longer be able to climb the ladder (score of 0).
4: Presence or absence of severe weight loss, lethargy, sometimes accompanied by tremors or seizures (moribund state).

5: Death

The clinical scores were determined from a weekly assessment of individual behaviors discussed in the previous section. The individual behavioral scores did produce a clinical score in an additive way, but rather indicated a ranking or level of disability displayed by that animal at that time. The graph in Figure 4.12 shows the mean clinical scores for all vector-immunized (19 mice) and all FP1-immunized (initially 90 mice, but diminishing with time as some died or were sacrificed) animals as a function of time. Ten animals (2 cages) from each FP1 immunization group were selected at random at the beginning of the study as a 'natural history' group. The behavioral data from this observation group along with all living mice at each time point provided large amounts of behavioral data used in the histograms in Figure 4.11 and the curves in Figure 4.12. It should be noted that the 'natural history' animals were not sacrificed for histopathology testing. The unimmunized control animals formed a 0 baseline score and are not shown in 4.12.

The control mice immunized with the induced vector-host lysate showed some deviation from normal behavior, but none attained a clinical score of 3 or greater unless the animal exhibited some other
behavioral characteristic such as extreme hyperactivity. The response to immunization with FP1 was more pronounced than for control immunizations signifying that the adjuvant, the GST tag and traces of bacterial proteins can illicit some behavioral changes such as temporary weight loss, decreased limb strength and poor rod and ladder testing. However, the more prolonged effects observed in FP1-immunized animals such as severe weight loss, loss of limb strength, and ataxia do not result from a response to the adjuvant or GST vector.

The duration of specific behaviors or characteristics was not monitored except that it was noted the following week whether the animal’s performance of each test had improved, remained stable or continued to deteriorate. Some animals fluctuated from week to week in rod and ladder test results, although complete loss of a skill was usually a permanent disability. Certain features of an animal’s gait could also fluctuate, but other characteristics, notably stride width abnormalities and ataxic gait were permanent disabilities. Animals that improved their behavioral test scores at 4 weeks post-immunization had gained weight, increased activity and normal handling responses. Most of these animals returned to nearly unimmunized control behavior scores.

Approximately 30% of the FP1-immunized animals in all dosage groups except the 200µg remained affected clinically and histologically abnormal to some degree as a result of reactivity to the FP1 immunogen.
A dose response effect is not clearly seen for the FP1 dosage groups at least in part because the 100μg FP1 group was immunized with a 5-fold higher amount of adjuvant additive, H37RA. This renders the host response to the immunogen more efficient (Section 2.9).

The animals that maintained the highest clinical scores were in the 1mg and 100μg dosage groups, but there were severely affected animals in all dosage groups. Most of these affected animals reached an average clinical score of 3 and stabilized at that level of disability. The animals that reached a higher clinical score either died or their condition was stabilized with a modified feeding regimen.

Overall, the immunized animals regardless of dose group, became lethargic, and less responsive to handling. Some animals developed other characteristics such as aggression in the form biting when handled or when their feet were being inked, or persistent irritating behaviors to their cage mates. These may reflect sensory abnormalities, but no association to other behavioral tests was found.

It was noted that several animals in the 1mg FP1 dose group became partially or totally blind. This kind of impairment would be expected to affect other tests where a visual prompt is important. An example of this is the "visual cliff" test as described in Section 4.8.1(Crawley and Paylor, 1996). This was observed in several mice in the 1mg dose group and a couple of mice in the lower dose.
Hyperactivity can also cause a mouse to fall off an edge, and this was the case with one mouse in the vector immunized control group. Blind mice also did not like the confinement of the walkway and therefore appeared to have an erratic gait. On the stationary rod, these mice would either cling onto the rod, remaining frozen in one position or they would slip off the rod easily.

4.10 Histopathology

Histological characterization of the disease process in the central nervous tissues was key to determining whether inflammation had established itself in the brain and spinal cord. This was done using 2 techniques: Hemotoxilin and Eosin staining (H & E) of fixed paraffin-embedded tissue and immunohistochemistry of fast frozen tissue with lymphocyte and macrophage-specific primary antibodies and horseradish peroxidase (HRP)-conjugated secondary antibodies. The H & E method is a high contrast visible stain of the cell nuclei in blue (hemotoxilin) and the surrounding cytoplasm in bright pink (eosin). Aggregates of mononuclear cells or cells with abnormal morphologies are clearly visible with this stain.

None of the animals in these large group studies were perfused with a fixative. All animals were perfused with either PBS or Ringer's
Solution, and the brains were divided along the corpus callosum. One half of the brain was fast frozen in dry ice-cooled isopentane as described in Sections 3.13 and 3.14 and the other half was fixed in Formalin at room temperature. The latter fixed tissue was processed for H & E staining by Histotechniques (Powell, OH). The fast frozen tissue was sectioned in 8 micron horizontal thick sections for immunohistochemical staining as described in section 3.14.

A histology scale was adapted from Pitt, et al. (2000). These authors rank their histological findings according to the number of layers of lymphocytes present in an area of inflammation. Each area of inflammation was scored, and the scores were added together for each lesion.

**Histopathology Scale**

0: no change

1: perivascular/submeningeal inflammation 1 cell layer thickness (slight).

2: perivascular/ submeningeal inflammation of greater than 1 cell layer thickness (extensive).

3: parenchymal staining (pervasive).
Each animal was given an additive score based on whether any one, two or all three kinds of inflammation were present in a particular section (described above). The scores were summed for 2 sections, and the mean was calculated. Therefore, the highest score possible was six.

Examples of the kind of histopathology observed with H & E staining in histologically positive animals is shown in Figure 4.13. The panel marked Control is of a portion of the cerebellum, and the oval hole in the middle of the field is a normal blood vessel in cross section. The two right hand panels marked Week 4 and 5 are also cerebellar sections. The large white-centered ovoid in the upper right panel is an inflamed blood vessel in cross section. The multi layers of dark mononuclear cells are the infiltrating inflammatory cells creating a ‘cuff’ like appearance. This degree of inflammation would be given a score of 2 on the histopathology scale. The lower right panel is the same thing except that this blood vessel is cut at a slightly transverse angle giving it a tear drop shape. The lower left panel marked Week 4 is of the hippocampus. This is a region more or less in the center of the brain where there is a channel between the parts of the hippocampus. Large aggregates of mononuclear cells like this were found in the hippocampus of several animals. This animal would also be given a score of 3 because there are areas of inflammation 1 cell thick along with this large area of mononuclear cell aggregation. Spinal cords and the cauda equina were
sectioned in a few representative animals, but no inflammation was observed. The cauda equina represents peripheral nervous tissue.

4.11 Immunohistochemistry

As the name implies, immunohistochemistry stains cells or cell structures by means of antibodies to markers that may be specific for cell type, developmental stage or pathological state. The method is best done with unfixed, fast frozen tissue. This is an extremely flexible technique that allows the use of many combinations of primary antibodies -- monoclonal or polyclonal antisera both work well. The secondary antibodies also allow for several types of visualization or detection that will alter the kind of information obtained and the sensitivity of the technique. While immunofluorescent secondary antibodies are more sensitive, they have the disadvantage of not being stable over long periods of time. Also, this is generally an ‘absence vs. presence’ method of detection meaning that it is most effective for seeing if a positive signal is present or absent. It does not provide information about the exact location of the signal or the signal fits into the whole tissue.

A comprehensive listing of the antibodies used in this study of the CNS tissues is in Appendix A. In this case, 3 T cell-specific monoclonal
primary antibodies were used – anti-CD3+, CD4+ and CD8+. CD3+ is expressed on both CD4+ cells and CD8+ cells, and was used primarily to pre-screen tissue sections before proceeding with the other two T cell monoclonals. The fourth primary antibody was the rat monoclonal F480 which is specific for activated macrophages and microglia.

The advantage of monoclonals is that they are epitope-specific; the disadvantage is that they are all made in rodents, most often in mouse. This creates the 'mouse-on-mouse' situation where background staining of residual IgG in mouse tissue sections can be high. For this reason, the Vector MOM kit was frequently used to block brain and spinal cord sections and then probe them with mouse monoclonal antibodies (Vector Labs, Burlingame, CA). This proved completely effective under the conditions described in Section 3.14. All brain sections were stained in parallel with a control section that tested for secondary antibody background staining, and all were negative (not shown).

Several searches were made of antibody databases to obtain an antibody for activated C3d that is specific for activated complement, but none was found. The C3 antibody used in this study is capable of detecting both activated and precursor forms of complement. Interestingly, the only animals that stained positively with C3 were immunized either with FP1 or with the induced vector lysate. The FP1-immunized animals demonstrated widespread staining throughout the
parenchyma of the cerebellum compared with slight parenchymal staining in the vector-host immunized animals (not shown). However, this staining was not optimized as it is unknown whether the antibody is also picking up non-activated C3.

Most of the animals that stained positively with any antibody other than F480 were also positive with the F480 antibody. Macrophages were present particularly in perivascular and meningeal areas as well as the parenchyma of the cerebellum. This staining was a consistent feature of most of the animals with inflammation (Figure 4.14) indicating a strong role for activated macrophages in this experimental encephalitis.

CD3+ and CD4+ T lymphocytes were detected in intense clusters in the meninges. Two animals immunized with 100μg of FP1 stained with anti-CD8+. One had meningeal aggregates of CD8+ T lymphocytes and another showed finely widespread CD8+ staining associated with the blood vessels in the cerebellum extending into the parenchyma (Figure 4.14). All control animals, both unimmunized and immunized with the vector-host lysate were negative for all H & E staining and immunohistochemistry (Figure 4.15). The spinal cord tissue from control or FP1-immunized animals did not show positive staining nor did the cauda equina stain which represents peripheral nerve tissue.
4.12 Relationship of Clinical and Histological Findings

Scatter plots were made of the histological and clinical scores of individual animals at weekly intervals following immunization with FP1 (Figure 4.16). The scatter plots indicate variation between individual clinical and histological scores. Nonetheless, the animals in the 1mg and 100µg dose groups show the most response to immunization.

The scores for the individual animals from the scatter plots were used to graph the clinical scores and histopathology scores separately as a function of time (Figure 4.17). The curves are shown superimposed in order to show the relationship of histopathology and the clinical phenotype as a function of time. The individual points on these curves are the averaged values for the 3 FP1 dosage groups from Figure 4.16. This is to say that only those animals selected for sacrifice and histological analysis are shown in figures 4.16 and 4.17. This is a subset of the FP1-immunized animals shown in the graph in Figure 4.12. However, in both instances, the clinical scores are mean values of animals from all dosage groups except that the sample size in Figure 4.17 is smaller as shown in Figure 4.16. There is a marked difference in mean clinical score on Day 7 between Figures 4.12 and 4.17. This is almost certainly due to the fact that some animals are badly affected by the first immunization. This situation causes them to be selected in a
biased manner for sacrifice to prevent losing the histological data from an animal that would likely die overnight. Therefore, the mean clinical score at Day 7 for the smaller sampling of mice in Figure 4.17 is 4 compared with a score of 2 in Figure 4.12.

The rapid rise of both curves by Day 7 reflects the onset of behavioral abnormalities in these mice in conjunction with the appearance of inflammation in the brain. The drop in both clinical and histological and clinical scores by Day 14 coincides with the week that the animals received their booster immunizations and likely indicates a subsidence of the initial response to immunization. Both curves rise 2-3 weeks post-immunization reflecting the peak response to re-immunization in incomplete adjuvant. By Day 42, there is a gradual decrease and leveling off of the clinical scores. The clinically affected FP1 mice showed little change in their behavioral tests by 6 weeks post-immunization. The level of histopathology observed in the animals remained fairly constant with no indication of remissions or relapses.

Approximately 30% of animals immunized with FP1 were histologically and clinically symptomatic. The onset of clinical manifestations of the autoimmune encephalitis described in this study coincides with the appearance of inflammation in the brain as indicated in Figure 4.17. Only those animals with grade 2 or higher histopathology developed grade 3 or higher clinical scores. Animals with an
intermediate clinical score of 2 did not necessarily have histopathology in the FP1-immunized animals. Several of the vector-immunized control animals reached this clinical score and none of them had detectable inflammation.

The areas of inflammation were highly reproducible in animals with positive histopathology, perivascular, meningeal and cerebellar staining. Evaluating the degree of inflammation was only approximated in that only 2-4 sections/animal were quantitated and these were not serial sections. If sections from an animal did not show histological abnormalities, it did not mean that none were present. Inflammation may have been present, but not seen in the slides examined. Therefore, associations between the presence of inflammation and clinical phenotype could not be made 100% of the time. It is important to note that more significant than degree of inflammation is the location of inflammation and the activated cells types contributing to the inflammation.

The strongest area of macrophage and complement reactivity was the cerebellum (Figure 4.14), and this was consistent with the gait abnormalities observed in these animals. T lymphocyte staining was always associated with perivascular and meningeal tissues. These are regions accessed by the cerebrospinal fluid which how activated T lymphocytes enter the central nervous system.
The resulting phenotype of this autoimmune encephalitis is a monophasic disease characterized by initial weight loss, progressive muscle weakness and disruption of gait. The histopathology indicates the presence of activated macrophages and microglia particularly in the cerebellum and the presence of mononuclear cells associated with the meninges and perivascular tissues.
Figure 4.1. Reamplified RT-PCR Product of FP1.

Lane 1 is a negative PCR control containing FP1 sense and antisense primers, but no template DNA. Lane 2 is the 1kb Plus DNA ladder (Life Technologies, Rockville, MD). Lane 3 is the 407bp FP1 PCR product reamplified from first round RT-PCR.
Figure 4.2. DNA Alignment of the FP1 RT-PCR Product and the Rat SCN2a Template.

The top line designated FP1 is the DNA sequence of the RT-PCR product cloned from rat brain total RNA. The bottom line is the template sequence of the rat brain type 2 voltage-gated sodium channel (SCN2a). The dark shaded boxes are the bases that differ in the template relative to the FP1 PCR product. Six base pair substitutions occurred, but only the $g$ to $a$ substitution at position 115 in the FP1 PCR product produced an amino acid change (Val311-Met).
Figure 4.2
**Figure 4.3.** Amino Acid Alignment of the FP1 Region of Central Nervous Sodium Channels.

The top sequence is the FP1 PCR product amino acid sequence. The sequences labeled R indicate rat sodium channels, M is murine, and H is human. SCN2a2 is a splice variant of SCN2a, and SCN3a is the embryonic form of the axonal voltage-gated sodium channel. The amino acids in dark shaded boxes are non-identical amino acids relative to highly conserved sequences. The star indicates the single amino acid change (Val311→ Met) introduced by a g to a base substitution at position 115 in the FP1 PCR product.
Figure 4.3
<table>
<thead>
<tr>
<th>BALB/c Strain</th>
<th>[M.tuberculosis] (H37RA) μgm</th>
</tr>
</thead>
<tbody>
<tr>
<td>[FP1] μgm</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>X</td>
</tr>
<tr>
<td>50</td>
<td>No pathology</td>
</tr>
<tr>
<td>100</td>
<td>Weight loss Hindlimb weakness Poor grooming</td>
</tr>
<tr>
<td>200</td>
<td>Hindlimb weakness Poor grooming</td>
</tr>
</tbody>
</table>

Preliminary Dosages of FP1 in Complete Freund’s Adjuvant (BALB/c strain)

Table 4.1
<table>
<thead>
<tr>
<th>SJL/J Strain</th>
<th>[M. tuberculosis] (H37RA) µgm</th>
</tr>
</thead>
<tbody>
<tr>
<td>[FP1] µgm</td>
<td>10</td>
</tr>
<tr>
<td>50</td>
<td>X</td>
</tr>
<tr>
<td>100</td>
<td>General weakness</td>
</tr>
<tr>
<td>200</td>
<td>Hind limb weakness</td>
</tr>
<tr>
<td></td>
<td>Hind limb weakness</td>
</tr>
<tr>
<td></td>
<td>Hind limb weakness</td>
</tr>
<tr>
<td></td>
<td>Hind limb weakness</td>
</tr>
<tr>
<td></td>
<td>Hind limb weakness</td>
</tr>
</tbody>
</table>

Preliminary Dosages of FP1 in Freund’s Complete Adjuvant (SJL/J strain)

Table 4.2
Figure 4.4. Western Blot of FP1-GST Fusion Protein Probed with Sera from Immunized and Control BALB/c and SJL/J Mice.

Lane 1. FP1-GST probed with pooled, adsorbed sera from FP1-immunized mice (1/1000 dilution). Lane 2. FP1-GST probed with pooled, unadsorbed sera from unimmunized control SJL/J mice (1/1000 dilution). Lanes 3 and 4. FP1-GST probed with pooled, adsorbed sera from SJL/J mice immunized with FP1-GST (1/1000 dilution).
Figure 4.5. Western Blots of the FP1-GST Fusion Protein and the Full Length Rat Type 2 Voltage-gated Sodium Channel.

Bottom panel: Lane 1. Induced FP1-GST fusion protein probed with pooled SJL/J sera from FP1-immunized mice (1/1000 dilution). Lane 2. Uninduced FP1-GST probed with pooled SJL/J sera from FP1-immunized mice (1/1000 dilution). Lane 3. Induced pGEX vector only (no insert) probed with commercial anti-GST antibody (1/4000 dilution; Apbiotech, Piscataway, NJ). Lane 4. Induced FP1-GST probed with commercial anti-GST antibody (1/4000 dilution; Apbiotech, Piscataway, NJ).

Top panel: An 8% SDS-PAGE of the CNalla cell lysate was run using visual markers (Rainbow Markers; Apbiotech, Piscataway, NJ) in order to separate the large proteins. Lane 1. Probed with pooled SJL/J sera from FP1-immunized mice (1/1000 dilution). Lane 2. Probed with pooled BALB/c sera from FP1-immunized mice (1/1000 dilution). Lane 3. Probed with commercial polyclonal anti-type 2 voltage-gated sodium channel antisera (1/500 dilution; Research Diagnostics Inc., Flanders, NJ). Lane 4. Probed with vector-immunized SJL/J pooled sera (1/1000 dilution). Lane 5. Probed with unimmunized SJL/J mouse control sera (1/1000 dilution). Lane 6. Goat anti-mouse-HRP secondary antibody control sera (1/2000 dilution; Jackson Immunologicals, West Grove, PA).

The antibodies used for western blotting are summarized in Appendix A.
Figure 4.5
Figure 4.6. Immunofluorescence of the CNaIIa Cell Line.

The CNaIIa cell line is stably transfected with the full length rat type 2 voltage-gated sodium channel (SCN2a) in the Zn**-inducible vector pZEM. The left panel is a semi-confluent culture of uninduced CNaIIa cells stained with adsorbed, pooled sera from FP1-immunized BALB/c mice. The right panel is a semi-confluent culture of induced CNaIIa cells stained with adsorbed, pooled sera from FP1-immunized BALB/c mice. The secondary antibody is goat anti-mouse IgG FITC (Jackson Immunochemicals, West Grove, PA).
Figure 4.7. SDS-PAGE of the FP1-GST Fusion Protein for Immunizations.

The 12.5% polyacrylamide gel was stained with Coomassie Brilliant Blue R-250. The dilutions of the FP1-GST fusion protein are: Lane 1. 2mg; Lane 2. 1mg; Lane 3. 500 μg; Lane 4. 250μg; Lane 5. 100μg; Lane 6. 50μg.
**Figure 4.8. Weight Changes in Immunized and Unimmunized SJL/S mice.**

The weights of the animals for all dose groups were calculated and the mean taken at regular time points. The squares (■) represent the weight curve for the unimmunized (normal) control SJL/J mice. The triangles (▲) are the mean weights for the vector-immunized control mice. The diamonds (◆) are the mean weights for the FP1-immunized mice.
Figure 4.8

Days Post Injection

Mean Weight (grams)
Figure 4.9. Gait Evaluation of Control and FP1-immunized SJL/J Mice.

The top panel shows the stride of a normal mouse. The rear paws are inked black and the forepaws are inked red. The stride length is indicated by the horizontal (red) line; the stride width is indicated by the perpendicular (blue) line. All measurements were done from the center of the footpad of the hind paw to the center of the same hind footpad in front (length) or to the opposing hind footpad (width). The lower panel is an example of an irregular stride where the front and rear paws are no longer a consistent distant apart. Also, both stride length and width are irregular.
Figure 4.9

Normal Mouse

Ataxic FP1-immunized Mouse
**Figure 4.10.** Walkway for Gait Testing.

The narrow walkway is approximately 2 feet long, 1 inch wide and 4 inches high so that the mice cannot turn around during testing or jump out of the walkway. A length of cash register tape cut in half lengthwise is used to line the walkway for each walk recorded.
Figure 4.11. Histograms of Individual Behavioral Tests.

The black bars indicate the normal (control) behavior for each test. The light bars indicate a behavior associated with the affected phenotype for each test. C labels the unimmunized control mice; VC indicates the control mice immunized with the induced pGEX vector lysate. The numbered bars refer to dose of FP1 used to immunize the mice (in micrograms). An affected gait indicates loss of symmetry of stride length and/or width. Abnormal performance on the stationary rod is defined as an inability to move or remain on the rod. The animals with abnormal ladder climbing skills did not climb the ladder more than half way to the top and were unable to reverse direction when the ladder was turned 180°. Fore limb strength was abnormal if the forepaws were unable to hold onto the wire cage top while the animal was pulled backwards by the base of the tail. Hind limb strength was diminished if the animals was unable to hang by its hind legs from the rim of its cage. Additional information concerning behavioral test analysis is listed in Appendix B.
Figure 4.11

163
Figure 4.12. Clinical Scores Attained by Immunized Control and FP1-immunized SJL/J Mice.

No curve was plotted for the unimmunized control mice as they all had a clinical score of 0. The squares (■) represent the changes in the mean clinical scores of the vector-immunized control mice. The circles (●) are the mean values of clinical scores for all dose groups of FP1-immunized mice. The error bars indicate standard error. The clinical ranking scores and individual behavioral test scores are listed in Appendix B.
Figure 4.12
**Figure 4.13.** H & E Staining of Perfused Brain Tissue.

The Control section in the upper left is of the cerebellum of a normal, unimmunized SJL/J mouse. The oval hole in the center of the section is a blood vessel in cross section with a normal monolayer of nuclei lining the inside surface of the vessel. The upper right panel is a cerebellar section of an FP1-immunized SJL/J mouse 4 weeks following immunization. A blood vessel is shown in cross section with 2-5 layers of infiltrating mononuclear cells. The lower left panel is an area of intense mononuclear infiltration extending from several blood vessels into the parenchyma of the surrounding cerebrum. The lower right panel is a cerebellar section of an FP1-immunized SJL/J mouse 5 weeks following immunization. The teardrop-shaped object in the center of the field is an oblique view of a blood vessel with 3+ layers of vascular infiltration.
Figure 4.14. Immunohistochemistry of Brain Tissue from FP1-immunized SJL/J Mice.

The upper left panel is the outer edge of the cerebellum stained with anti-CD3 monoclonal antibody. The intense area of staining is a submeningeal inclusion of CD3+ lymphocytes. The upper right panel shows 2 small submeningeal inclusions of CD4+ lymphocytes stained with monoclonal anti-CD4 indicating the presence of helper T cells in this region of the cerebellum. The lower left panel shows an area of intense staining with a monoclonal anti-CD8 antibody. The CD8+ cytotoxic T cell staining follows a line of blood vessels between the folia of the cerebellum. The lower right hand panel shows strong submeningeal staining of the cerebellum using the f480 monoclonal antibody specific for activated macrophages and microglia. Details of antibody dilutions and commercial sources are tabulated in Appendix A.
Figure 4.15. Immunohistochemistry and H & E Staining of Brain Tissue from Unimmunized and Vector-immunized Control SJL/J Mice.

Panel A shows a cerebellar section stained with H & E of a control mouse immunized with uninduced pGEX vector/XL-1 bacterial lysate. The large white ovoid in the lower center and the small white circle in the upper center of the section are normal blood vessels. Panel B is a cerebellar section also from a vector-immunized control mouse stained with monoclonal antibody f480. No specific staining is observed. Panel C is the hippocampus region of an unimmunized control mouse stained with H & E. Note the normal monolayer of cells lining the inner surface of the ventricle.
**Figure 4.16.** Scatter plots of clinical and histological scores from individual FP1-immunized SJL/J mice at weekly intervals.

Summary clinical and histological scores were plotted for individual mice at weekly intervals. The black circles (●) are the 100μg animals; the triangles (▲) are the 200μg dosage, and the open circles (○) are the 1mg dose group. The plots indicate a range of variance between the two values at all time points except on Day 14 when there is greater clustering of points indicating a closer association. Overlapping points have a figure 8 appearance. The scales for histological and clinical ranking are listed in Appendix B and discussed in Sections 4.9 and 4.10.
Figure 4.16
Figure 4.17. Summary Graph of Histological and Clinical Scores of Selected FP1-immunized SJL/J Mice.

A curve represented by the circles (●) is the mean histological score for selected FP1-immunized mice from the scatter plots in Figure 4.16. The clinical scores for these mice are indicated by the squares (■). This shows that the development of disability by the FP1-induced autoimmune encephalitis is associated with histopathology in the brains of these animals. The bars indicate the standard error at each time point.
CHAPTER 5

DISCUSSION OF RESULTS AND CONCLUSIONS

The murine model of central nervous sodium channel-induced encephalitis presented in this dissertation is a first step towards addressing the effects of axonal autoimmunity in the CNS. The animals immunized with the FP1 fusion protein of the type 2 voltage-gated sodium channel developed a monophasic disease where most of the affected mice stabilize at a moderate level of disability. This is not simply an acute response to immunization as vector-immunized animals experience some weight loss and temporary behavioral changes without the chronic disability or any of the histopathology that is observed in FP1-immunized mice.

The FP1-induced disease is characterized by the presence of macrophages widely spread throughout the cerebellum. Meningeal and perivascular infiltration of mononuclear cells and severe mononuclear infiltration of the hippocampal region is also a feature of this experimental encephalitis. Small, intense clusters of CD4+ T cells were
also observed in the cerebellum most often associated with blood vessels and the meninges. CD8+ T cells were less frequently observed, but were present in the meninges and blood vessels and cerebellar parenchyma. The timing of immune cell infiltration relates to the onset and clinical phenotype of the animals. The topography of inflammation is best characterized by abnormal balance and co-ordination characteristics on a stationary rod, and development of an abnormal gait.

The expression of autoantibodies to FP1 was universal for FP1-immunized stains of mice. The BALB/c strain, however, appears resistant to the encephalitogenic effects of FP1. This is not unlike other attempts to induce EAE with myelin antigens in the BALB/c strain. Only those SJL/J mice that also developed histopathology displayed clinical disability. The SJL/J strain is known to be particularly susceptible to experimental autoimmune diseases, and this is the case for the sodium channel autoantigen described in this dissertation.

Thirty percent of susceptible animals develop symptomatic encephalitis which, in this case, is characterized by moderate to severe weight loss, moderate to severe muscle weakness and loss of co-ordination typified by an ataxic gait and/or an inability to balance on a stationary rod. A small, but reproducible number of affected animals (approximately 15-20%) have additional neurological findings such as seizures, trembling, or blindness. In rare instances, an animal died
within 2 weeks of immunization. This was probably either due to severe weight loss and dehydration and/or severe neurological disease observed in these animals, but probably not due to a specific response to FP1.

Demyelination and spinal cord pathology were noticeably absent from this autoimmune encephalitis. Sensory defects were not rigorously assessed, but virtually all affected mice, and some unaffected animals showed marked lethargy and changes in transfer evoked behaviors. These observations were specific for FP1-immunized animals and were more pronounced in the animals with the affected phenotype.

This first demonstration of central nervous sodium channel-induced autoimmune encephalitis is important because it specifically targets an axonal protein. Existing EAE models target proteins specific to glial cells and astrocytes. While current models of autoimmune encephalomyelitis have contributed a great deal to the current level of understanding of neuroimmunology, they fall short of addressing what is occurring in the nerve axon in inflammatory demyelinating diseases such as MS. If the disability in MS patients is truly a reflection of neuronal loss, then it is important to study the disease from the perspective of the axon as well as the surrounding glia.

Many questions remain regarding the cellular and molecular pathology of this sodium channel EAE such as: can the disease be passively transferred by activated T lymphocytes to naïve syngeneic
animals? Also, will injections of sodium channel autoantibodies induce disease in susceptible animals? Considering the large size of the sodium channel it is feasible that other determinants may be more encephalitogenic than the FP1 region. These should be investigated either with fusion proteins as discussed here or with synthetic peptides to these epitopes. Considering the role the β subunits play in intra and intercellular communication, these may also provide a source of additional epitopes that are important to the disease process. It is encouraging that the α subunit of the sodium channel is located in regions found to be genetically associated with multiple sclerosis on human chromosome 2.

More in depth characterization of the experimental encephalitis presented here needs to be done. For example, studies characterizing axonal changes that may contribute to the development of the affected phenotype should be undertaken using confocal microscopy. These studies may provide some insight concerning immune-mediated axonal degeneration.

Finally, it is my hope that the work presented here opens a door to new perspectives and possibilities in the field of neuroimmunology. Further development of axonal models of disease will increase the understanding of mechanisms underlying immune-mediated neurodegenerative diseases. Also, the experimental autoimmune
"channelopathy" described in this dissertation provides an additional model system for the study of other diseases where autoantibodies to channels are present either as a primary or secondary aspect of the disease. Examples of this include Stiffman Syndrome, myasthenia gravis and Lambert-Eaton Syndrome.

Only in the last 15 years or so, has the level of technology and scientific understanding made it possible to study multi-factorial diseases like MS. It is important to look at such complex disease from many perspectives and glean observations from widely differing approaches. The explosive amount of accessible data from molecular genetics and immunology is making it possible to bring together some pieces of an elusive puzzle. Animals models of diseases such as the one presented here bring together many lines of investigation allowing several aspects of multi-factorial diseases to be studied in a complete biological system. Some of the lessons are always the same, and that is that in vivo studies are never easy to interpret one hundred percent of the time. On the other hand, there are, to date, no better or more fruitful experimental systems in which to study complex human disease.
REFERENCES


C. Davenport (1922) Multiple sclerosis from the standpoint of geographic distribution and race. *Archives of Neurology* 8:51-8.


J. Lakshmanan (1978) Nerve growth factor induced turnover of phosphatidyl inositol in rat superior cervical ganglia. *Biochemical and Biophysical Research Communications* 82:767-75


Appendix A

Antibody Tables
<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Final dilution</th>
<th>Secondary Antibody</th>
<th>Product #</th>
<th>Storage</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c adsorbed serum pool</td>
<td>1:250 4°C overnight</td>
<td>sheep anti-mouse IgG-HRP, goat anti-mouse IgM-HRP, goat anti-mouse IgG+IgM-HRP</td>
<td>Sigma A-6782 Jaxn 115-035-020 Jaxn 115-035-044</td>
<td>4°C, -20°C glycerol</td>
<td>1:3000 1:5000</td>
</tr>
<tr>
<td>SJL adsorbed serum pool</td>
<td>1:250 4°C overnight</td>
<td>sheep anti-mouse IgG-HRP, goat anti-mouse IgM-HRP, goat anti-mouse IgG+IgM-HRP</td>
<td>Sigma A-6782 Jaxn 115-035-020 Jaxn 115-035-044</td>
<td>4°C, -20°C glycerol</td>
<td>1:3000 1:5000</td>
</tr>
<tr>
<td>SJL serum pool</td>
<td>1:500 4°C overnight</td>
<td>sheep anti-mouse IgG-HRP, goat anti-mouse IgM-HRP, goat anti-mouse IgG+IgM-HRP</td>
<td>Sigma A-6782 Jaxn 115-035-020 Jaxn 115-035-044</td>
<td>4°C, -20°C glycerol</td>
<td>1:3000 1:5000</td>
</tr>
<tr>
<td>SJL uninjected serum pool (NEG. CONTROL)</td>
<td>1:500 4°C overnight</td>
<td>sheep anti-mouse IgG-HRP, goat anti-mouse IgM-HRP, goat anti-mouse IgG+IgM-HRP</td>
<td>Sigma A-6782 Jaxn 115-035-020 Jaxn 115-035-044</td>
<td>4°C, -20°C glycerol</td>
<td>1:3000 1:5000</td>
</tr>
<tr>
<td>CNalla injected SJL serum pool (ADJUVANT CONTROL)</td>
<td>1:500 4°C overnight</td>
<td>sheep anti-mouse IgG-HRP, goat anti-mouse IgM-HRP, goat anti-mouse IgG+IgM-HRP</td>
<td>Sigma A-6782 Jaxn 115-035-020 Jaxn 115-035-044</td>
<td>4°C, -20°C glycerol</td>
<td>1:3000 1:5000</td>
</tr>
<tr>
<td>Rabbit SP19 Chemicon AB5210a</td>
<td>1:2000 4°C overnight</td>
<td>goat anti-rabbit IgG-HRP</td>
<td>Sigma A-4914</td>
<td>4°C</td>
<td>1:4000</td>
</tr>
<tr>
<td>Rabbit SP1 RDI NACHAN9abr</td>
<td>1:500-1:1000 4°C overnight</td>
<td>goat anti-rabbit IgG-HRP</td>
<td>Sigma A-4914</td>
<td>4°C</td>
<td>1:4000</td>
</tr>
<tr>
<td>Goat anti-GST Pharmacia</td>
<td>1:2000 1 hr RT</td>
<td>rabbit anti-goat IgG-HRP</td>
<td>Sigma A-5420</td>
<td>4°C</td>
<td>1:3000</td>
</tr>
</tbody>
</table>

Table A1. ANTIBODIES FOR WESTERN BLOTS
<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Product #</th>
<th>Storage</th>
<th>Dilution</th>
<th>Secondary Antibody</th>
<th>Product #</th>
<th>Dilution</th>
<th>Blocking</th>
</tr>
</thead>
<tbody>
<tr>
<td>rat anti mouse F480 Mab</td>
<td>Serotec MCAP497</td>
<td>-20º C or 4º C</td>
<td>1:500</td>
<td>rabbit anti-rat IgG-biotin *mouse adsorbed donkey anti-rat IgG-HRP donkey anti-rat IgG-rhodamine (TRITC)</td>
<td>Vector Labs BA-4001 Jxn 712-035-153 Jxn 712-025-153</td>
<td>1:100 (2.5µg/ml)</td>
<td>PBS/5% normal rabbit 1hr</td>
</tr>
<tr>
<td>mouse anti mouse CD3+ Mab</td>
<td>Pharmin gen 15261A</td>
<td>4º C</td>
<td>1:250</td>
<td>goat anti-mouse IgG-HRP goat anti-mouse IgG Cy-3</td>
<td>Jxn 115-035-062 Jxn 115-165-006</td>
<td>1:2500</td>
<td>MOM block 1:1 PBS/5% normal goat 1 hr</td>
</tr>
<tr>
<td>mouse anti mouse CD4+ Mab</td>
<td>Pharmin gen 01061D</td>
<td>4º C</td>
<td>1:500 (1µg/ml)</td>
<td>goat anti-mouse IgG-HRP goat anti-mouse IgG Cy-3</td>
<td>Jxn 115-035-062 Jxn 115-165-006</td>
<td>1:2500</td>
<td>MOM block 1:1 PBS/5% normal goat 1 hr</td>
</tr>
<tr>
<td>mouse anti mouse CD8+ Mab</td>
<td>Pharmin gen 01041D</td>
<td>4º C</td>
<td>1:250 (1µg/ml)</td>
<td>goat anti-mouse IgG-HRP goat anti-mouse IgG Cy-3</td>
<td>Jxn 115-035-062 Jxn 115-165-006</td>
<td>1:2500</td>
<td>MOM block 1:1 PBS/5% normal goat 1 hr</td>
</tr>
<tr>
<td>goat anti mouse complement C3</td>
<td>Cappel/ICN 55444</td>
<td>-20º C 1:1 glycerol</td>
<td>1:1600</td>
<td>rabbit anti-goat IgG-biotin donkey anti-goat IgG-HRP rabbit anti-goat IgG-TRITC</td>
<td>Life Tech Jxn Sigma T-7026</td>
<td>1:4000</td>
<td>PBS/10% normal rabbit 1hr</td>
</tr>
</tbody>
</table>

Table A2. ANTIBODIES FOR IMMUNOHISTOCHEMISTRY
Appendix B

Behavioral Test Scoring System
LIST OF INDIVIDUAL BEHAVIORAL TESTS

Hind Limb Strength

0: slips off edge; cannot hang on
1: hangs for a period of time, drops off edge
2: pulls out onto cage rim and walks along

Forelimb Strength

0: no strength
1: slight, slips off grid with gentle tug
2: active, effective grasp

Ladder Climbing

0: cannot go up ladder; may fall off
1: can partially climb the ladder, but cannot reorient when ladder is turned
2: can climb to the top and reorient when the ladder is turned 180° (normal)

Stationary Rod

0: falls off rod in less than 2 seconds, no grip, no balance
1: unsteady, cannot regain control
2: unsteady but regains control for more than 10 seconds
3: remains in one position on rod, perhaps unsteady
4: rights itself and can walk along the length of the rod using tail as a limb

Righting Reflex

0: absent
0.5: present but mouse splays limbs on contact with mat
1: present
**Grooming**

0: no grooming, scruffy coat, dirty bottom, limbs, or face, odor  
1: inky scruffy coat, dirty bottom, odor  
2: some ink on fur (mostly underside)  
3: clean, soft, fluffy white, no odor

**Tail Curl**

0: cannot curl tail or curls tail >25% from tip  
1: curls tail at 25% from tip  
2: curls tail at 40% or more from tip (normal)  
EK: touch-evoked kink in tail  
PK: kink in tail always present

**Provoked Biting**

0: absent  
1: bites when provoked w/ Q-tip

**Aggression**

0: absent  
1: provoked biting aggression

**Fear**

0: absent  
1: freezes upon transfer arousal

**Tremors**

0: none  
1: mild  
2: marked tremors
Other Behaviors During Gait Testing

0: normal fluid movement (or normal for that mouse)
1: keeps moving, but not fluid; stops & goes, turns around
2: halting, waddling (ataxic), hopping, dumping
3: panicked, runs off end of walkway
4: may be visually impaired, walks off walkway

Torso Curl

0: Absent; wags head; flailing
1: inward (abdominal) curl
2: sideways curl; nose toward tail
3: sideways curl; nose reaches tail

Limb Grasping

0: Absent
1: partial grasping; in and out; grasps one set of limbs (1F or 1H)
2: grasps both sets of limbs tightly

Circling

0: Absent
1: runs around perimeter of cage
2: runs in a small vicious circle

Transfer Evoked Activity

0: no change from normal activity, less than 1 sec. Hesitation
1: hesitation then continues with normal activity
2: nervous and agitated
3: frenzied, thrashing
4: paralyzed, frozen
**Spontaneous Activity**

-1: slow to arouse, doesn’t interact  
0: none/normal activity  
1: vigorous grooming  
2: rapid darting  
3: extremely vigorous, almost frenzied movement

**Unusual Behaviors**

A. Spontaneous/intermittent  
   jumping  
   flipping  
   trembling

B. Repetitive/reproducible  
   circling  
   flipping  
   trembling  
   humping  
   splat
**Clinical Assessment Scale (composite)**

0: No discernable changes in motor or behavior
1: Some hind limb weakness
2: Increased ascending weakness and/or diminished ladder climbing ability
3: Inability to remain on stationary rod and/or gait abnormalities; may no longer be able to climb the ladder
4: Severe weight loss, lethargy, sometimes accompanied by tremors or seizures
5: Death

Assign a score corresponding to the level of disability observed for individual behavioral tests contributing to the composite score. The highest score at one time point is 5. Score for each animal may fluctuate weekly.

**Histopathology Ranking Scale (composite)**

0: no change
1: perivascular inflammation 1 cell layer thickness
2: perivascular inflammation of greater than 1 cell layer thickness
3: parenchymal staining

Calculation is made by ADDING the score for each type of lesion observed per section. Take the mean of 2 sections. Highest possible score is 6. The animal was sacrificed in order to obtain histology, therefore, the calculated score for that animal will not change.
Appendix C

Clinical and Histological Data Charts
Gait Evaluation Chart

<table>
<thead>
<tr>
<th>Date</th>
<th>Stride Length (L)</th>
<th>Stride Length (R)</th>
<th>Stride Width</th>
<th>Total Strides</th>
<th>Fall off Walkway</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mouse #:  
Cage:  
Perfused:  
Dose:  
Day:  

Date:  
Stride Length (L)  
Stride Length (R)  
Stride Width  
Total Strides  
Fall off Walkway  
Comments 

218
### Behavioral Test Chart

<table>
<thead>
<tr>
<th>Mouse #:</th>
<th>Dose:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cage:</td>
<td>Day:</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Date</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pencil</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forelimb strength</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hindlimb strength</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grooming</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Circling</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Torso Curl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Limb Grasp</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Righting Reflex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ladder</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tail Curl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Immunohistochemistry of Mouse Tissue

<table>
<thead>
<tr>
<th>Week</th>
<th>Mouse</th>
<th>F480</th>
<th>C3</th>
<th>CD3</th>
<th>CD4</th>
<th>CD8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>AEC</td>
<td>AEC</td>
<td>AEC</td>
<td>AEC</td>
<td>AEC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TRITC</td>
<td>TRITC</td>
<td>TRITC</td>
<td>TRITC</td>
<td>TRITC</td>
</tr>
</tbody>
</table>

---

220